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The Effect of Rigid Sodium Restriction in Patients With Cirrhosis of the Liver and Ascites. W. J. Eisenmenger, M.D., E. H. Ahrens, Jr., M.D., S. H. Blondheim, M.D., and Henry G. Kunkel, M.D., New York, N. Y. ---- 1029

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THE EFFECT OF RIGID SODIUM RESTRICTION IN PATIENTS WITH CIRRHOSIS OF THE LIVER AND ASCITES

W. J. Eisenmenger, M.D., E. H. Ahrens, Jr., M.D., S. H. Blondheim, M.D., and Henry G. Kunkel, M.D.
New York, N. Y.

LOW salt diets have been used for many years in the treatment of edema and ascites in patients with cirrhosis of the liver. Enthusiastic reports appeared in the French literature as early as 1904 claiming actual termination of ascites formation from the use of a low salt diet.[1, 2, 3] In recent years, however, emphasis on high protein, high caloric diets in treatment of cirrhosis has led many physicians away from the use of low salt regimens, since it has seemed difficult to devise a palatable low salt diet which is adequate in protein. Moderate restriction of salt has continued to be common practice in the treatment of ascites but results have not been striking.

Recently, numerous reports have appeared dealing with the role of sodium in the formation of ascites and edema in cardiac disease.[4, 5, 6] Sodium and water retention by the kidney has been claimed as a factor in the production of ascites in cirrhosis as well as in congestive heart failure by Farnsworth.[7] Layne and Schemm[5, 8] have applied this principle to the management of cardiac and cirrhotic ascites by means of low salt diets. Chalmers and Davidson[9] have shown in a preliminary study of sodium balance in cirrhosis that salt plays an important role in fluid retention. Recently, the work of Whipple and coworkers[10] on dogs rendered ascitic by constriction of the inferior vena cava above the liver has demonstrated that ascites of purely mechanical origin can be affected markedly by variations in salt intake.

The role of hypoalbuminemia in the formation of ascites in patients with liver disease has been recognized by numerous observers.[11, 12] Ralli and coworkers[13] have obtained evidence that increased excretion of antidiuretic substance also plays a role in ascites formation. Recently, the work of Blakemore[14] has demonstrated the importance of portal hypertension as a third factor in the formation of ascites. The present report represents a description of the direct relationship between the sodium content of the diet and the accumulation of ascites in thirteen patients despite the operation of the other three factors. Detailed studies of electrolyte balance in three of these patients will be published in a separate communication.

MATERIALS AND METHODS

The thirteen patients to be discussed were admitted to the Hospital of The Rockefeller Institute at least ten days prior to the initiation of salt restriction in the diet. The diagnosis

From the Hospital of The Rockefeller Institute for Medical Research.
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of cirrhosis of the liver was established by characteristic history, numerous physical examinations, x-ray studies of the esophagus and abdomen, and a wide variety of liver function tests. Ascites had been present for four to forty-eight months prior to the onset of low salt therapy and had necessitated two to seventy-eight paracenteses. Fluid continued to accumulate at the usual rate at the time of onset of therapy.

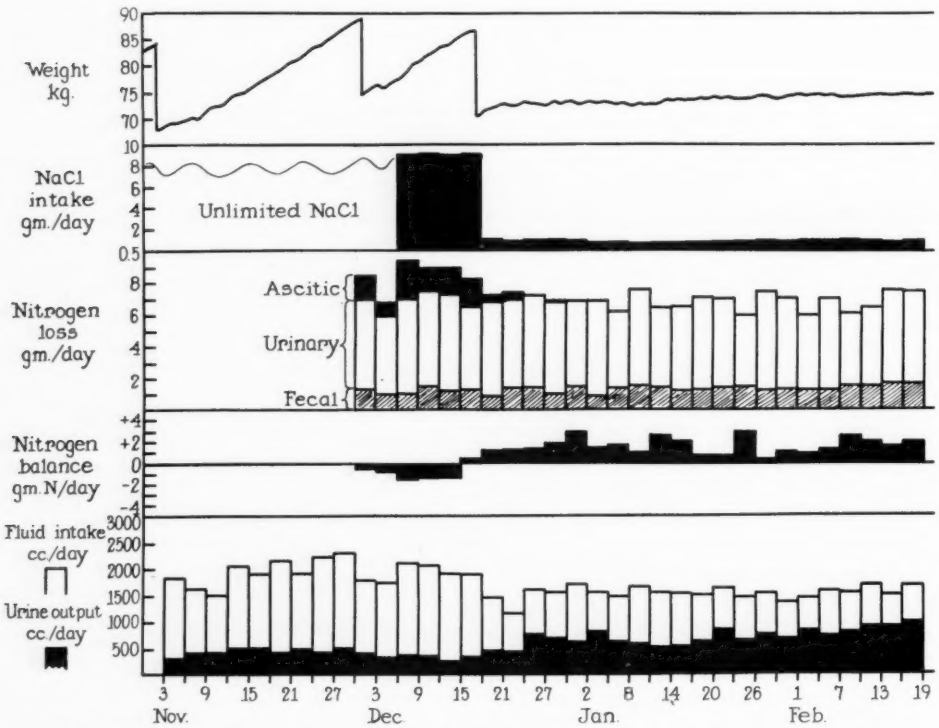

Fig. 1.—Case 1. Three paracenteses in the hospital prior to Na restriction. Immediate cessation of ascites formation on diet of 1 Gm. of NaCl per day. Reversion from negative to positive nitrogen balance.

The etiology of the cirrhosis was chronic alcoholism with associated nutritional disturbance in nine of the thirteen patients. None of the patients was suffering from an enlarged, acutely decompensated fatty liver at the time of admission.

The diets used were prepared by trained dietitians who kept careful records of the daily intake of protein, fat, carbohydrate, and salt. An effort was made to provide patients with an excess of food and calculations were made from the amounts offered and refused. Protein, salt, and caloric intake were calculated from standard tables. The sodium intake was checked by direct analyses. In the detailed studies on sodium balance, direct analysis of the sodium content of the food, urine, and feces was carried out. (NaCl was calculated on the basis of measurements of Na.) In the low salt diets, special attempts were made to keep the food palatable. Salt-free bread, available for purchase, was used. No salt substitutes were administered. Where protein intake tended to be low, or where a high protein, low sodium intake was sought, whole protein supplements were added to the diet.* As much as 50 Gm. of protein per day could be provided with these palatable supplements. Serum albumin was determined by a modification of the specific immunologic method of Chow.[15] Instead of measuring turbidity, the antigen-antibody precipitate was determined by means of the ninhydrin reagent of Moore and Stein.[16] The Howe method with Kjeldahl digestion

*Delcos Granules (Sharp & Dohme); Melactin (Squibb); Protinal (National Drug).

was also used for protein determination. Osmotic pressures were determined by a specially constructed Hepp type osmometer. Determinations of serum bilirubin, bromsulfalein retention, thymol turbidity, and total lipids were also carried out in all of the patients during the period of therapy. The methods employed were identical with those presented in a previous study.[17] Plasma chlorides were determined by the method of Van Slyke[18]; plasma volume, by the method of Gibson and Evans.[19] Sodium was determined by means of a Perkin-Elmer flame photometer utilizing lithium as an internal standard.

RESULTS

CASE 1.—A 52-year-old man with cirrhosis of unknown etiology beginning six months prior to admission and requiring six paracenteses. Physical examination showed occasional spider angiomata and a small, hard liver. Laboratory tests indicated an advanced cirrhosis. The total protein was 5.7 Gm. per cent, with albumin 1.9 Gm. per cent. Bromsulfalein retention was 37 per cent in 45 minutes. Serum esterase was 22 units. Cephalin flocculation was 2+; the thymol turbidity was 10 units and bilirubin was 1.8 mg. per cent.

On admission the patient continued to accumulate ascites at a regular rate (Fig. 1), necessitating three paracenteses while on an unlimited NaCl intake. This was found by analysis to average 9.2 Gm. NaCl per day. Urinary excretion of NaCl was extremely low, averaging 0.04 Gm. per day, while fecal NaCl was 0.3 Gm. per day. The patient was in negative nitrogen balance on the normal diet. This was chiefly due to the loss of ascitic nitrogen through the paracenteses.

Immediately following the initiation of a low salt diet which on analysis was found to contain between 0.8 and 1.2 Gm. NaCl per day, the ascites formation ceased and the patient reverted to positive nitrogen balance as indicated in Fig. 1. The urinary output approximately doubled and both the urinary and fecal NaCl concentrations fell. The serum and ascitic fluid Na levels showed a fall of approximately 3 meq. per liter. After the patient remained on this diet for three months, the urinary NaCl which had remained constant suddenly increased and the patient lost considerable ascites. Table I illustrates the sudden rise in the urinary NaCl despite continued NaCl restriction. The dietary intake improved progressively during this time and positive nitrogen balance increased. At the present time, six months after the initiation of Na restriction, the patient is markedly improved although not entirely recovered. He now tolerates 4 and 5 Gm. NaCl per day without forming ascites because of the increased urinary output of Na.

The total protein in the serum and ascitic fluid rose by 2 Gm. per cent after five months on the diet.

TABLE I

	NACL GM./DAY			NA MEQ./LITER	
DATE	FOOD	URINE	FECES	SERUM	ASCITIC FLUID
Dec. 3	9.1	0.05	0.30	138	137
Dec. 15	9.3	0.07	0.35	139	139
Jan. 12	1.1	0.02	0.15	135	135
Feb. 12	0.9	0.02	0.12	135	134
Mar. 24	1.0	2.00	0.25	137	137
May 12	0.9	1.00	0.18	136	135

Comment.—Rigid NaCl restriction caused a cessation of ascites formation, positive nitrogen balance, and a rise in serum and ascitic fluid proteins. Urinary NaCl increased with the mobilization of ascitic fluid. A five-month period of therapy was necessary before the patient showed signs of recovery.

CASE 2.—Fig. 2 illustrates the course of a 42-year-old woman with a nutritional cirrhosis who had ascites for eight months prior to admission. She had a total of twenty-nine paracenteses in that period, during which time she was at home on minimal activity. Since an

accurate record of these was kept, they are indicated in the weight curve of Fig. 2. Her diet, poor at the onset of the ascites, was adequate for six months prior to admission. The skin showed numerous spider angiomata and prominent liver palms. The liver edge was barely palpable at the costal margin. An esophagram showed esophageal varices. Laboratory tests likewise pointed to an advanced cirrhosis. Proteins formed by the liver were all low; plasma albumin was 2.4 Gm. per cent, fibrinogen 225 mg. per cent, prothrombin

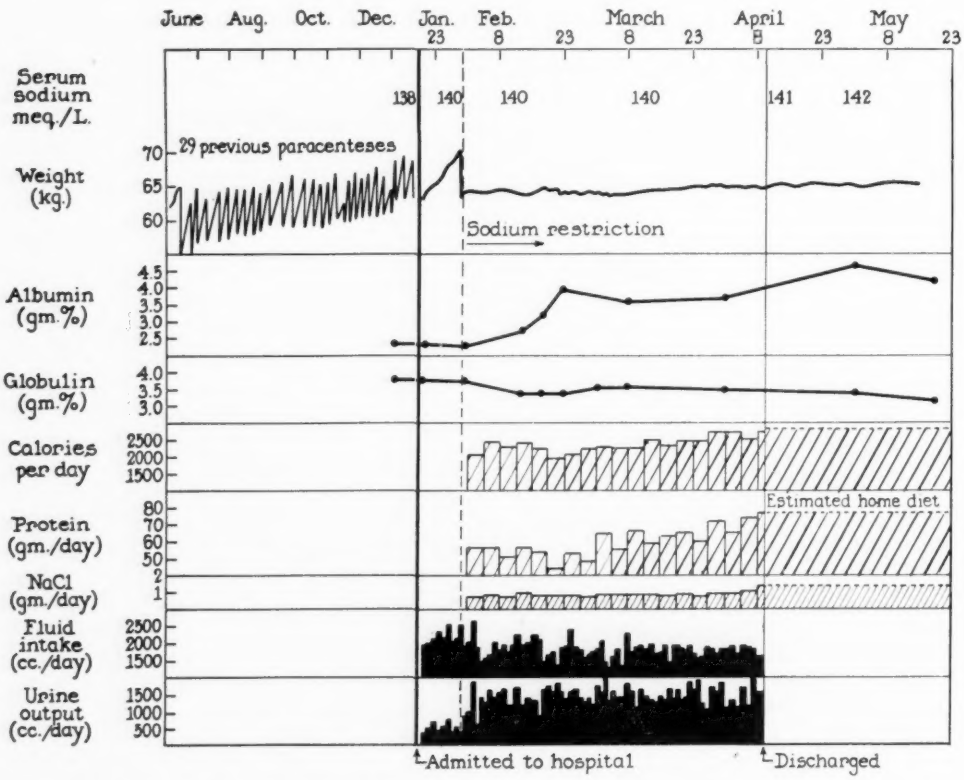

Fig. 2.—Case 2. Ascites of eight months' duration requiring thirty paracenteses. Immediate control of ascites with salt restriction. Prompt increase in urinary output and delayed rise in serum albumin.

38 per cent of normal, and esterase 13 units. Gamma globulin was elevated as indicated by a zinc turbidity of 32 units and a toal serum globulin of 3.5 Gm. per cent. Bromsulfalein retention was 40 per cent in 45 minutes. Ascitic fluid total protein was 1.0 Gm. per cent, with albumin 0.7 Gm. per cent.

On admission the patient was placed on a regular hospital diet and activity equivalent to that at home. On this regimen she continued to gain weight and ascites at her usual rate. The urinary output was small. Following a paracentesis which became necessary, she was put on a diet with NaCl restricted to 1 Gm. per day. The response was immediate with a complete cessation of ascites accumulation and a rise in urinary output to about three times the previous output. This occurred despite a spontaneous decrease in fluid intake. A small amount of ascites, detectable by shifting dullness, persisted. This effect was sustained throughout her hospitalization and at home. After three months the patient was able to tolerate larger amounts of NaCl in her diet without forming ascites. She has remained well during the year since discharge and all ascites has disappeared.

A rise in serum albumin from a subnormal level of 2.4 Gm. per cent to normal levels of 4 to 4.5 Gm. per cent occurred.

Clinically there was marked improvement in this patient. As soon as she became adjusted to the diet, she complained only of the flat taste of food without salt but this did not interfere with her eating a perfectly adequate diet. The termination of her need for paracenteses after having had thirty was a strong factor in raising her morale and insuring cooperation.

Comment.—A low salt diet aided in restoring this patient to a fairly normal, active life, after having previously required almost weekly paracenteses for seven months. The urinary output likewise showed an immediate increase, while the serum albumin levels gradually rose to almost double the former levels within three weeks.

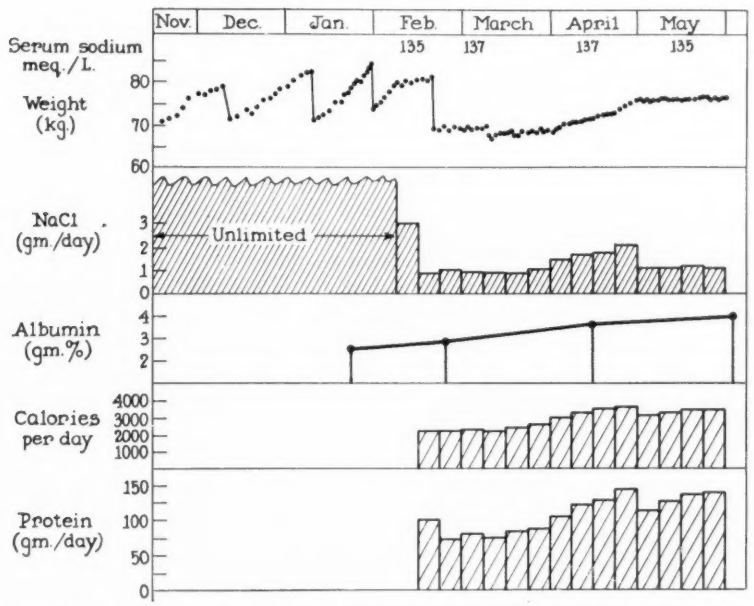

Fig. 3.—Case 3. Ascites formation controlled by NaCl restriction at 1 Gm. per day. Reaccumulation of ascites on 1.5 to 2 Gm. NaCl per day. Control of ascites again at 1 Gm. per day. High protein intake (140 Gm. per day) despite salt restriction to 1 Gm. per day.

CASE 3.—Fig. 3 illustrates the course of a 54-year-old wine drinker who on admission to the hospital was accumulating ascitic fluid at a regular rate. He had been hospitalized elsewhere on two occasions. Following an unexplained febrile episode he lapsed into hepatic coma for four days but gradually recovered. Three paracenteses were performed at the other hospital following the episode of coma. Since weights were available, these are illustrated in the weight curve of Fig. 3. On physical examination the patient showed a small, hard liver and marked ascites with no edema. Following admission to the Rockefeller Hospital he was kept on a regular diet with normal sodium intake for three weeks. Ascitic fluid continued to accumulate and a fourth paracentesis was performed. Following the reduction of the NaCl content of his diet to 3 Gm. per day, there was an immediate slowing in the rate of ascites formation as is readily visible from the weight curve of Fig. 3. The NaCl content was further reduced to approximately 1 Gm. per day, and following a fifth paracentesis the patient ceased accumulating ascitic fluid for one month. The NaCl content of the diet was then gradually raised to 2 Gm. per day. Ascitic fluid began to accumulate and the weight curve illustrated in Fig. 3 can be seen to rise with progressive increments

of salt intake. When NaCl was reduced again to 1 Gm. per day, ascitic fluid again ceased accumulating and the weight curve flattened out. By means of protein supplements, as much as 140 Gm. of protein were supplied a day during this latter period despite the very low NaCl intake.

Improvement on the low salt regimen was very striking. The discontinuance of paracenteses caused the patient to feel better and to eat better and resulted in a rise in the albumin concentration in the serum. The limitation of salt was very well tolerated. After three months on the low sodium diet, the patient recovered sufficiently to tolerate a normal diet at home without ascites.

Comment.—The degree of salt restriction necessary to control the ascites in this patient was well defined at about 1.3 Gm. a day. Limitation below this level promptly resulted in a favorable therapeutic response with control of ascites and a marked increase in urine volume. By the use of protein and carbohydrate supplements it was possible to raise the patient's protein intake to 140 Gm. per day and his caloric intake to over 3,000 calories despite limitation of sodium chloride to 1 Gm. per day.

CASE 4.—Fig. 4 illustrates the course of a 60-year-old housewife who developed hepatic cirrhosis without known cause and had been steadily accumulating ascites for four years. She had had seventy-six paracenteses prior to admission, with removal of an average of 10 liters per tap followed by leakage of fluid from the wound for one to two weeks. After closure of the wound she consistently accumulated approximately 1 liter of ascitic fluid per day. Edema of the legs appeared as the intra-abdominal pressure increased.

In the nine months prior to admission the patient had become progressively weaker. She had lost considerable body tissue and had had several episodes suggesting minor cerebrovascular accidents. In the course of the four years the patient had been hospitalized for all paracenteses, and the main therapeutic efforts were directed toward a highly nutritive diet with occasional trials of mercurial diuretics.

On admission she appeared chronically ill, with marked tissue wasting. The liver was slightly enlarged and hard. Laboratory findings were consistent with an advanced cirrhosis with a decrease in the proteins formed by the liver; serum albumin was 2.5 Gm. per cent, prothrombin level 47 per cent of normal, fibrinogen 160 mg. per cent, serum esterase 27 units. Bromsulfalein retention was 23 per cent. Ascitic fluid total protein was 1.1 Gm. per cent, with albumin 0.5 Gm. per cent.

On the hospital diet with unrestricted salt the patient continued to gain weight and ascites at her usual rate until two paracenteses were performed. Just before the second tap in the hospital, salt in the diet was limited to 1 Gm. a day. The rate of accumulation of ascites was promptly slowed and eventually leveled off at a point just short of requiring a paracentesis. With the institution of the low salt diet, urine output which had been minimal (200 to 300 c.c. a day) except following mercurials approximately doubled. This occurred despite a voluntary reduction in fluid intake. Attempts to raise the fluid intake were futile, leading to nausea. This may have been due to impaired renal function presumably on an arteriosclerotic basis. Both prior to and during the period of sodium restriction the patient showed a constantly elevated nonprotein nitrogen, a urea clearance of 25 per cent, and urine specific gravity fixed at 1.010. The patient's diet averaged 60 Gm. of protein and 2,000 calories daily, with NaCl limited to below 1 Gm. a day.

Laboratory tests showed no change with the exception of a moderate rise in serum albumin levels, from 2.5 Gm. per cent to 3.7 Gm. per cent. During the entire course there were but insignificant variations in the hemoglobin levels. Clinically this patient improved for two months but then her dietary intake fell and she was returned to a normal diet. A second period on the low Na diet again proved of little permanent value and the patient died three months after returning to the normal Na intake.

Comment.—The chronicity of this patient's illness with resultant debility and prolonged ascites plus the renal and cerebral arteriosclerotic complications provided a difficult test for this regimen. After seventy-eight paracenteses the rate of ascites formation was promptly slowed by salt restriction, but the dietary intake was poor and the patient eventually died.

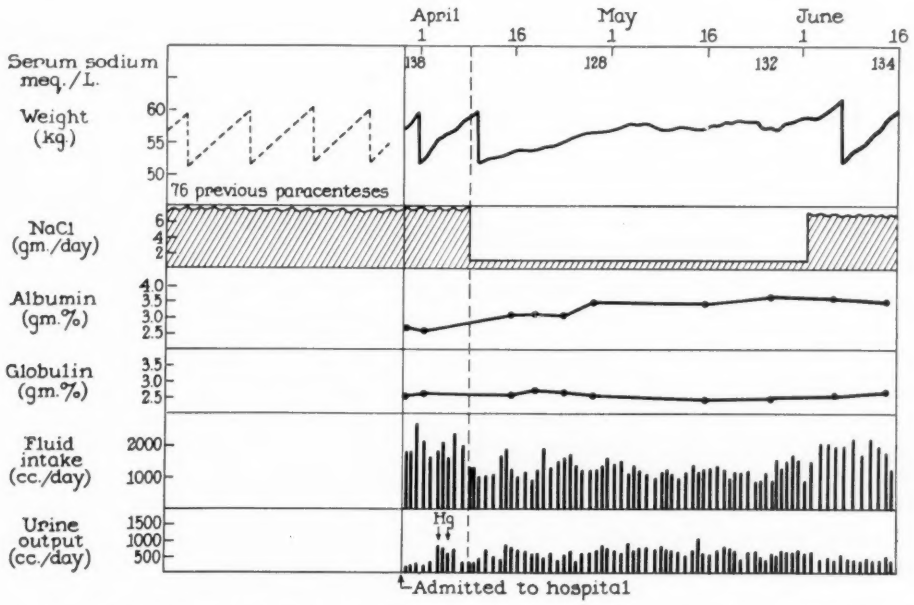

Fig. 4.—Case 4. Ascites of four years' duration requiring seventy-eight paracenteses. Effect of salt restriction in controlling formation of ascites. Increase in urine output and serum albumin level. Return of ascites and need for paracenteses on normal diet.

DISCUSSION

The results of rigid Na restriction have been described in detail in four of the thirteen patients studied. The remainder of the patients showed similar results with complete cessation of ascites formation when the NaCl was limited to 1 Gm. per day. Higher intakes caused ascites formation in direct proportion to the NaCl given. An average critical level of NaCl intake was obtained for these patients above which ascites formation occurred. This was 1.2 Gm. NaCl per day. The reason for this direct relation between NaCl intake and ascites was that the NaCl excretion in the urine was extremely low regardless of how much NaCl was furnished in the diet. The urinary NaCl excretion in eight of these patients on a diet of 7 to 9 Gm. NaCl per day ranged from 0.02 to 0.13 grams. Fecal NaCl excretion was higher, averaging 0.35 Gm. per day. With this low excretion of NaCl it is apparent that the large part of the NaCl intake is used to form ascitic fluid. Insensible NaCl loss was not measured but could be calculated from the NaCl balance data to be approximately 0.8 Gm. per day.

Excretion of water rose in all of the patients on the low sodium diet. When there was no Na supplied in the diet for ascitic fluid accumulation, water was

excreted proportional to the intake. A high fluid intake had little effect in altering daily sodium excretion.

All but one of the thirteen patients were able to tolerate the low Na regimen for at least three months without ill effects. The one exception, Patient 4, showed a fall in fluid and caloric intake on the diet. Another patient developed muscle cramps after being on the diet for five months. These were relieved by supplying Na. The patient had done well on the diet without forming ascites prior to the onset of the cramps. Serum Na levels were followed in all of the patients. A fall in serum Na of approximately 3 meq. per liter usually occurred. One patient who received mercurials in addition to the low Na diet showed the greatest fall, 12 meq. per liter.

Nitrogen balance studies were carried out in two of the patients. These patients were in negative balance prior to Na restriction because of the loss of ascitic nitrogen. When ascites formation ceased on the low Na diet, the patients reverted to positive nitrogen balance.

All of the patients demonstrated a rise in serum proteins on the low Na diet. Fig. 5 shows a group of determinations on the serum and ascitic fluid of one of the patients (Case 1). The rise in albumin and in colloid osmotic pressure was approximately equal in the two fluid compartments, demonstrating the equilibrium which exists. The total protein of serum and ascitic fluid rose considerably more than the albumin. Plasma volume determinations showed little change and thus did not account for the rise. Some of the other patients demonstrated a 10 per cent fall in plasma volume accounting for a portion of the protein rise. The patients who responded best to the diet and gained body weight soon after treatment was started showed a rise in serum albumin that was sometimes greater than the total protein rise. This was due to a fall in globulins. The selective rise in albumin was a sign of improvement in liver protein synthesis. This did not occur in the chart shown (Fig. 5) nor in most of the other patients. The rise in these patients of all the protein components appeared to be the result of cessation of protein loss in the ascitic fluid permitting the proteins to accumulate in the body.

The use of newly available low Na protein supplements permitted the administration of as much as 150 Gm. of protein per day on the low Na diet. The patients showed considerable individual variation in their ability to take different supplements, but of those which were used there usually was one which was readily tolerated by each patient. Improvement appeared to be more rapid when the protein was kept above 100 grams. However, some of the patients responded well at a level of 70 Gm. of protein per day.

The general clinical results of a three-month period on the low NaCl diet were evaluated by studying the effect of normal Na intake on ascites at the end of the three months. Four patients recovered sufficiently during this time to enable them to excrete the increased Na and they did not reform ascites. These patients gradually absorbed the remaining ascitic fluid and returned to a normal life. Eight patients were still unable to excrete larger amounts of Na and accumulated ascites on the normal diet. These patients had not regressed during the three months of Na restriction and all of them appeared

slightly improved but they had not been cured. Na restriction was reinstituted in these individuals. Three of these have since showed increased Na excretion representing loss of ascitic fluid after more than six months of therapy. Others in the group are still being treated with combined albumin, liver extract, and low Na therapy. One of these patients died of esophageal hemorrhages. Patient 4 who did not tolerate the low Na diet also died.

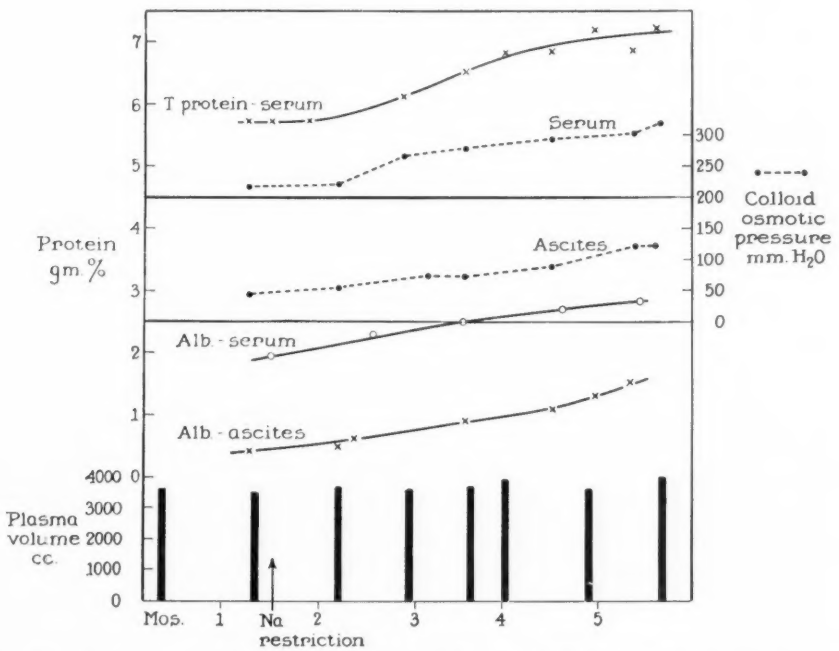

Fig. 5.—Rise in values for determinations of proteins and colloid osmotic pressure in serum and ascitic fluid following Na restriction.

In all of the patients the diet appeared to be beneficial in stopping the malignant course of events brought about by removal of protein through paracenteses in the already depleted individual, thus enabling dietary therapy to become effective. The long-term clinical results are difficult to evaluate but the fact that ascites formation could be completely controlled by rigid Na restriction was clearly evident.

SUMMARY

1. The results of rigid NaCl limitation in the diet of thirteen patients with cirrhosis of the liver and long-standing ascites are presented.

2. Ascites formation ceased for three months in twelve of the thirteen patients. Urine output increased in each case commensurate with decreased fluid retention.

3. Four patients did not reform ascites when a normal NaCl intake was tried after three months; eight patients did reform ascites.

4. Serum protein levels showed a rise following therapy. This is explained primarily on the basis of retention in the serum of protein which previously had been lost in the ascitic fluid.

5. A high protein and high caloric diet could be maintained despite rigid salt limitation.

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THE HYPERBILIRUBINEMIC EFFECT OF SODIUM NICOTINATE

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INTRODUCTION

NEW techniques are often instrumental in permitting important developments in the understanding of biological and clinical problems. In 1941, Villa[13] claimed that treatment with nicotinamide produced a striking remission of jaundice in different types of hepatic dysfunction, provided the liver damage had not reached the irreversible stage. Apparently while investigating the mechanism of this therapeutic effect, Mattei[9] observed that the injection of 30 mg. of sodium nicotinate intravenously in normal individuals was followed by a sharp rise of the level of indirect reacting bilirubin in serum. This technique has permitted interesting observations on some aspects of the metabolism of biliary pigments in normal and pathologic conditions.[8] The results obtained so far in more than twenty normal subjects and seventy-eight patients with various disorders of the liver will now be reported.

THE TECHNIQUE OF THE TEST

Ten cubic centimeters of blood are collected from the patient who has been fasting for the previous twelve hours, and 30 mg. of sodium nicotinate dissolved in 10 c.c. of saline solution are slowly injected through the same needle. The injection is followed almost immediately by a sensation of flushing and intense heat. Temporary marked vasodilatation is also observed, particularly in the face and extremities. Ten cubic centimeters of blood are again collected 90, 210, and 480 minutes after the injection. These intervals have been selected because they best show the changes of serum bilirubin level during the test, both in normal and pathologic conditions. Patients may be given a light meal after the third sample of blood has been collected, to avoid an excessively long period of fasting. This practice does not influence the result of the test.

The tubes containing the blood are left in the oven at 37° C. for one hour and the serum is then separated by centrifugation. To prevent hemolysis (which represents one of the major causes of error in the determination of the bilirubin level), the blood must be obtained with a perfectly dry syringe and needle,† by a clean venipuncture and without foaming. Total and direct reacting bilirubin are determined in each sample with the method of Jendrassik and Grof[6] and the value of bilirubin of the indirect reacting type is calculated by difference.

RESULTS

Effect of the Intravenous Injection of Sodium Nicotinate on Bilirubinemia.—Following the intravenous injection of sodium nicotinate in normal subjects the total bilirubin level of serum rises sharply, reaches a maximum value after ninety minutes, and then decreases slowly, returning to normal

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†Coating of syringes and needles with the film silicone (General Electric Dri-Film 9987) permits one to obtain serum free of hemolysis almost constantly. Technical details are given in a previous paper.[12]

figures within six to eight hours. Ordinarily the rise in circulating bilirubin is due to the "indirect reacting type" since no bilirubin of the direct reacting type appears in circulation. If the bilirubin variations are followed at regular

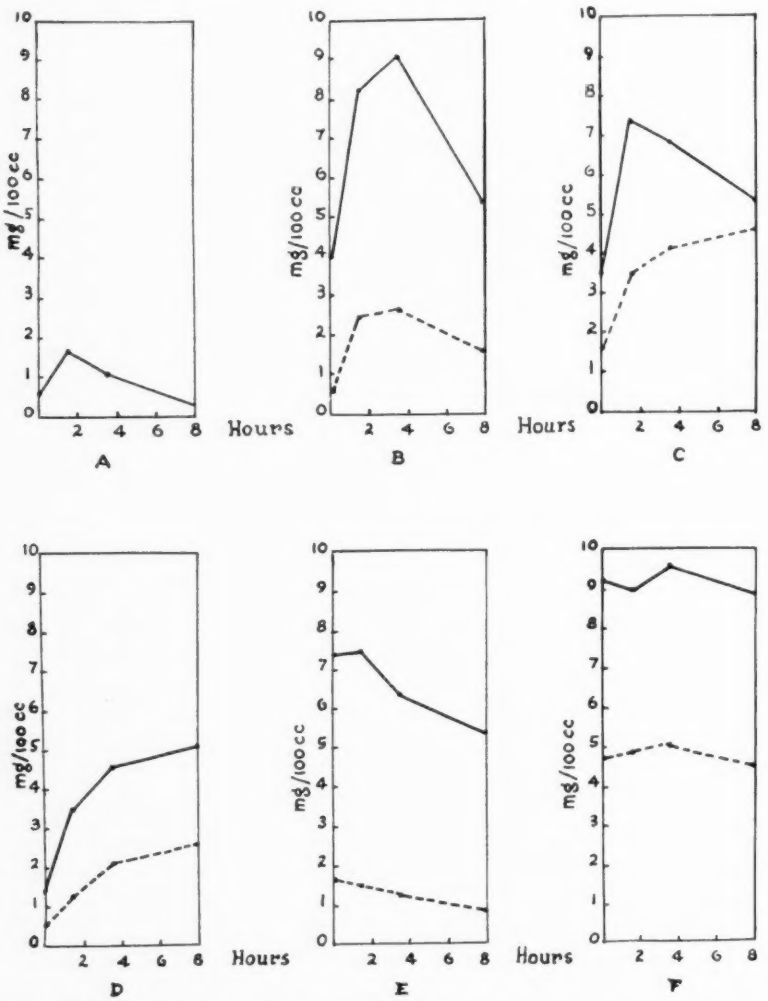

Fig. 1.—Different types of hyperbilirubinemic curves obtained in normal subjects and in patients with various liver disorders, following the intravenous injection of 30 mg. of sodium nicotinate.

(The continuous lines represent the level of total serum bilirubin; the broken ones, the level of serum direct reacting bilirubin.)

intervals and plotted against time, a curve is obtained with a sharp, short ascending and a prolonged, slowly descending segment. (Fig. 1, *A* shows an average normal curve, derived from the analytic values obtained in twenty-two normal subjects.) Serum cholesterol and bile salts are also influenced by the intravenous injection of sodium nicotinate (Table III), but so inconsistently that their variations do not present any clear significance. This phenomenon appears to be specific for sodium nicotinate. Chemically related substances,

TABLE I. LEVELS OF BILIRUBIN, CHOLESTEROL, BILE SALTS, CHLORIDES, AND URIC ACID OF SERUM IN A PATIENT WITH CIRRHOSIS OF THE LIVER, FOLLOWING THE INTRAVENOUS INJECTION OF SODIUM NICOTINATE (30 MG.)

TIME AFTER INJECTION	0 MIN.	90 MIN.	210 MIN.	480 MIN.
Total bilirubin (mg. per 100 ml.)	3.99	8.12	9.02	5.65
Direct reacting bilirubin (mg. per 100 ml.)	0.33	1.05	1.07	0.89
Cholesterol (mg. per 100 ml.)*	85	130	78	71
Bile salts (mg. per 100 ml.)†	0	20	8	0
Chlorides (Gm. per 100 ml.)	7.37	6.90	7.02	7.11
Uric acid (mg. per 100 ml.)	4.88	4.35	4.16	4.21

*Determined with the method of Autenrieth.
†Determined with the method of Cottet.

such as nicotinamide and nikethamide, and drugs sharing with sodium nicotinate some pharmacologic effects (histamine, epinephrine, and acetylcholine), either cause no modification of the serum bilirubin level or do so to a much lesser extent than sodium nicotinate (Table II). No significant changes of red blood cells and reticulocyte count (Table III) or of the blood level of chlorides and uric acid (Table I) follow the intravenous injection of sodium nicotinate.

TABLE II. EFFECT ON THE SERUM BILIRUBIN LEVEL OF THE PARENTERAL ADMINISTRATION TO NORMAL SUBJECTS OF SUBSTANCES CHEMICALLY OR PHARMACOLOGICALLY RELATED TO SODIUM NICOTINATE

SUBSTANCE	DOSE AND ROUTE OF ADMINISTRATION	SERUM BILIRUBIN LEVEL (MG.%) TIME AFTER INJECTION (MIN.) 0	30	90	180	210
Normal control		0.36		0.32		0.43
Sodium nicotinate	30 mg., intravenously	0.34	0.58	1.07		0.74
Histamine, hydrochloride	1 mg., intramuscularly	0.50	0.61	0.55	0.58	
Epinephrine, hydrochloride	1 mg., intramuscularly	0.70	0.86	0.67	0.59	
Acetylcholine, hydrochloride	100 mg., intramuscularly	0.48	0.53	0.51		0.50
Nikethamide	850 mg., intramuscularly	0.40	0.42	0.49		0.38
Nicotinamide	100 mg., intravenously	0.56		0.61		0.60

TABLE III. TOTAL SERUM BILIRUBIN LEVEL, RED BLOOD CELL AND RETICULOCYTE COUNT IN A NORMAL SUBJECT FOLLOWING THE INTRAVENOUS INJECTION OF 30 MG. OF SODIUM NICOTINATE

TIME AFTER INJECTION	0 MIN.	90 MIN.	210 MIN.	480 MIN.	24 HR.	48 HR.	72 HR.
Total serum bilirubin (mg. per 100 ml.)	0.75	1.40	1.05	0.89	–	–	–
Red blood cells (millions)	4.86	5.00	4.60	4.91	4.87	4.92	4.85
Reticulocytes (per thousand)	5	5	8	5	8	13	9

The Cholagogic and Choleretic Effect of Sodium Nicotinate.—When 30 mg. of sodium nicotinate are given intravenously to normal subjects in whom a Rehfuss tube has been passed previously into the duodenum, a copious flow of bile begins about ten minutes after the injection. For the first five minutes

the bile collected has the characteristics of bile A; then, for about twenty minutes, of bile B, and finally of bile C. The total amount of bile which is collected in one hour's time is 200 to 250 c.c., far greater than the amount obtainable through ordinary stimulation with the Meltzer-Lyon technique. If the serum bilirubin level is determined simultaneously, it appears to be only minimally increased. The same low type of hyperbilirubinemic curve is obtained when the biliary flow is stimulated with intraduodenal introduction of $MgSO_4$, thirty to sixty minutes before the intravenous injection of sodium nicotinate, and the bile obtained is, at least in part, extracted through the Rehfuss tube.

The first two findings suggest a direct stimulation of the bile-excreting function of the liver by sodium nicotinate. This has been, moreover, proved directly from the study of a patient suffering from chronic hepatocholangitis in which a surgical biliary fistula had been therapeutically established. This case, which offered a rare opportunity of following the modifications of the bilirubin level of bile as compared with those of serum bilirubin during the performance of the sodium nicotinate test, has been presented in detail elsewhere.[7] Notwithstanding the presence of the fistula, the concentration of stercobilin (determined in the feces with the method of Watson[15]) showed a considerable amount of bilirubin still flowing into the intestine. The injection of sodium nicotinate in this patient was followed by a limited rise in the serum bilirubin level. Its consequent decrease toward the initial value was accompanied in direct time relationship by an increase of bilirubin concentration in bile.

The Effect of the Intravenous Injection of Sodium Nicotinate on Urobilinuria.—The cholagogic and choleretic action of sodium nicotinate provides larger amounts of urobilinogen for reabsorption from the intestinal tract. This effect is further enhanced by the vasodilatation caused by the substance, particularly pronounced in the splanchnic area. A corresponding rise in the amount of urobilin eliminated could, therefore, be expected.

Studies on the changes in elimination of urobilin after the intravenous injection of sodium nicotinate have been carried out in twelve normal subjects and in a few patients with various grades of liver dysfunction. Urobilin level in urine has been determined with the original method of Heilmeyer and Krebs.[5] It has been found consistently that, following the intravenous injection of sodium nicotinate, the urinary concentration of urobilin rises to from five to eight times the original values, reaches a maximum value two to three hours after the injection, and then slowly returns to the normal level in twenty-four hours (Fig. 2, Table IV). This rise in concentration of urobilin is roughly proportional to that of the indirect reacting bilirubin of serum, although somewhat delayed. It is greatly pronounced (ten to fifteen times the original values) in patients presenting serious liver dysfunction.

In patients in whom direct reacting bilirubin appears or increases in circulation as an effect of the injection of sodium nicotinate, a contemporary appearance of bilirubin or rise in its concentration is observed in the urine.

TABLE IV. MODIFICATIONS OF THE LEVEL OF TOTAL AND DIRECT REACTING BILIRUBIN OF SERUM AND OF UROBILIN FOLLOWING THE INTRAVENOUS INJECTION OF 30 MG. OF SODIUM NICOTINATE

TIME AFTER INJECTION	0 MIN.	90 MIN.	210 MIN.	480 MIN.	24 HR.
(a) Subject M. S., ♂, age 29, normal					
Total bilirubin (mg. per 100 ml.)	0.22	0.97	0.68	0.19	—
Direct reacting bilirubin (mg. per 100 ml.)	0	0.18	0	0	—
Urobilin (mg. per 100 ml.)	0.042	—	0.526	0.372	0.058
(b) Patient N. H., age 48, ♂, suffering from chronic hepatocholangitis					
Total bilirubin (mg. per 100 ml.)	3.46	6.02	5.48	4.89	—
Direct reacting bilirubin (mg. per 100 ml.)	1.17	1.83	1.97	1.64	—
Urobilin (mg. per 100 ml.)	0.094	0.465	1.037	0.702	0.172

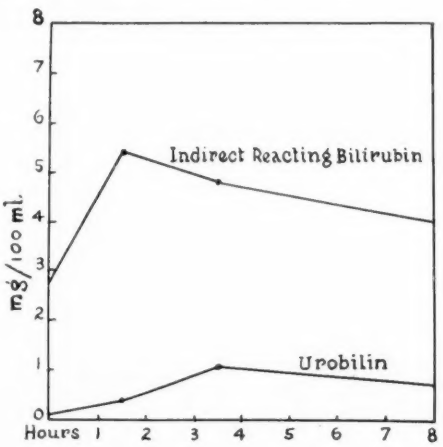

Fig. 2.—The effect of the intravenous injection of 30 mg. of sodium nicotinate on the level of serum indirect reacting bilirubin and the level of urobilinuria in a patient with chronic hepatocholangitis.

(The values of indirect reacting bilirubin and of urobilin are exprsesed in milligrams per 100 ml. of serum and urine respectively.)

Hyperbilirubinemic Curves in Normal and Pathologic Conditions.—As described previously, the intravenous injection of sodium nicotinate in normal subjects is followed by a rise in the level of indirect reacting bilirubin of serum, which returns to normal within eight hours.

The liver cell very probably disposes of the sudden load of indirect reacting bilirubin caused by the sodium nicotinate test by transforming it to direct reacting bilirubin and certainly eliminates the latter. A curve of the type shown in Fig. 1, *A* is therefore obtained when both bile-forming* and bile-excreting functions of the liver are normal. The curve is usually found: (a) in normal subjects, (b) in patients with moderately abnormal liver function but no clinical jaundice, (c) in a few patients presenting mild clinical jaundice and a serum bilirubin level slightly higher than normal. Some of the patients of the third group may also present a small amount of circulating direct bilirubin which is only insignificantly modified during the course of the test and always returns to the initial value at the end of the eight-hour period. Often, at the end of the

*To be understood as the capacity, probably possessed by the liver cell, of transforming the indirect to direct reacting bilirubin of elimination, and indicated by the gradual decrease of the circulating bilirubin of the indirect reacting type.

test, the total bilirubin level may be lower than the starting value, especially in normal subjects. It is possible that this effect is due to the stimulation of the bile-excreting function of the liver by sodium nicotinate, as has been previously discussed.

In pathologic conditions the hyperbilirubinemic curve may present two main modifications: (a) failure of the bilirubin level to return to normal values within the experimental period of eight hours; (b) appearance in circulation of bilirubin of the direct reacting type. Various combinations of these changes appear to be responsible for the different types of curve obtained in pathologic conditions. Two types are most commonly encountered. In the first (Fig. 1, *B*), values of total and direct reacting bilirubin increase sharply after injection of sodium nicotinate and decrease slowly without returning to the starting values at the end of the eight hours. In the second (Fig. 1, *D*), total and direct reacting bilirubin rise continuously from the moment of injection until the end of the experimental period. Both these curves indicate a serious impairment of the bile-excreting function of the liver (more serious, obviously, when the second curve is obtained) while the bile-forming function is still normal or, at least, less involved, as shown by the rise of circulating bilirubin of the direct reacting type. An extreme example of this condition is sometimes found (Fig. 1, *C*) when the indirect reacting bilirubin diminishes progressively but incompletely after the critical ascent in the first ninety minutes following the injection of sodium nicotinate, while the direct reacting bilirubin increases until the end of the test.

Other variations from the normal curve are less commonly found. In cases of jaundice due to arsenical poisoning a paradoxical effect may be observed. The values of total and direct reacting bilirubin remain unchanged for the first sixty to ninety minutes, then drop slowly (Fig. 1, *E*). This is probably due to the stimulating effect of sodium nicotinate on a sluggish but otherwise not greatly impaired bile-excreting function and explains the prophylactic and therapeutic activity of the substance in liver poisoning from drugs such as arsenobenzene.

Patients with complete obstruction of the biliary tract present a characteristic curve of flat type (Fig. 1, *F*), the significance of which will be discussed later.

DISCUSSION

For the purposes of interpretation, the hyperbilirubinemic curve due to sodium nicotinate must be considered separately in its two parts. The *descending one* is related to the bile-forming and bile-excreting function of the liver cell, as evidenced by the study of the patient with surgical biliary fistula, previously mentioned, in whom progressive decrease of the serum level of indirect reacting bilirubin was accompanied by a synchronous rise in concentration of bilirubin in the bile. The mechanism of the *ascending part* of the curve is, on the other hand, extremely difficult to interpret. It appears very improbable that the rise of the level of indirect reacting bilirubin is due to the mobilization of bile pigments from tissues because of the vasomotor effect of sodium nicotinate.

No tissue deposits of bile pigments are known to exist, at least in normal conditions,[1] nor is the injection of sodium nicotinate followed by changes in level of blood chlorides and uric acid (substances which are easily mobilized from tissues). Another possibility, namely that the hyperbilirubinemia may be due to an exaggeration of normal blood destruction, has no clear-cut experimental evidence in its favor. No changes of red blood cell and reticulocyte count follow the intravenous injection of sodium nicotinate, although these changes might be very small yet significant; (more important, they could be masked by variations of hemoconcentration). However, hyperbilirubinemic curves of normal type are usually obtained in patients with increased fragility of erythrocytes.*

Our findings suggest that the increase of indirect reacting bilirubin of serum is due to the increased reabsorption from the intestine of a biliary pigment (possibly mesobilirubin). This tentative conclusion is based on strong, but, so far, indirect evidence: (a) the powerful cholagogic and choleretic effect of sodium nicotinate, and (b) its pronounced vasodilatory action on the vessels of the splanchnic area. As a result, the reabsorption from the intestinal tract of components of bile is greatly increased. (Exaggerated reabsorption of a chromogenous bilirubin derivative—urobilinogen—following the intravenous injection of sodium nicotinate is shown by the corresponding changes in elimination of urobilin.) Further evidence shows that availability of bile in the intestine and hyperbilirubinemic effect of sodium nicotinate are directly related, as proved by two main findings: (a) the hyperbilirubinemic effect of sodium nicotinate is limited or absent in normal subjects when biliary flow is first stimulated by means of $MgSO_4$ and the secreted bile extracted as completely as possible by means of a Rehfuss tube passed into the duodenum; (b) patients with complete biliary obstruction do not present any hyperbilirubinemic response to sodium nicotinate.

The conclusion suggested is not supported by the accepted theories on the metabolism of biliary pigments. However, the Italian school of Zoja[16] has strongly claimed that serum bilirubin of the indirect reacting type represents reabsorbed mesobilirubin which has escaped the hepatic filter through lymph channels or the portacaval anastomoses. Notwithstanding the demonstration by Colangiuli[3] that mesobilirubin and indirect reacting bilirubin behave in a similar manner with various chemical reagents, the theory still awaits more conclusive evidence and is not generally accepted. It would, however, offer an explanation of our findings, since the increased flow of bile and the vasodilatation induced by sodium nicotinate could exaggerate the reabsorption of mesobilirubin† and consequently raise the serum level of indirect reacting bilirubin.

The increased elimination of urobilin which follows the intravenous injection of sodium nicotinate is another finding of clinical interest and practical

*A very low hyperbilirubinemic curve was observed in a patient with Werlhof's disease, recently splenectomized. This finding is difficult to interpret and thus does not specifically support or deny the view that exaggerated blood destruction is the cause of the hyperbilirubinemia due to sodium nicotinate.

†No hyperbilirubinemia follows the intravenous injection of nicotinamide, which is only slightly less active than sodium nicotinate in increasing the biliary flow but does not possess any vasodilatory effect. This indirect evidence would suggest that the hyperbilirubinemia depends more on the vasodilatory action than on the cholagogic and choleretic effect of sodium nicotinate.

importance. The effect is probably due to a double mechanism: (a) the presence in the intestinal tract of a large amount of urobilinogen available for reabsorption, due to the cholagogic and choleretic effect of sodium nicotinate; (b) the vasodilatory effect of the substance on the splanchnic vascular area, which increases the possibility of the chromogen being adsorbed directly into the general circulation through portacaval anastomotic vessels. The coordination of these two mechanisms explains the magnitude of the increased elimination of urobilin in the urine after intravenous injection of sodium nicotinate. From a clinical viewpoint the observation shows that *large quantities of urobilin may appear in the urine even when no liver damage or hyperhemolysis can be demonstrated.* This last finding indirectly confirms earlier experiments of Melli[10] who was able to show in dogs that the rise of pressure within the portal system (determined with experimental occlusion or direct compression of the portal vein, or pneumothorax of the right side) causes increased elimination of urobilin. When the pressure within the portal tree is increased, an amount of urobilinogen greater than normal reaches the general circulation and is finally eliminated as urobilin. A similar mechanism can be cited to explain the pronounced urobilinuria of clinical conditions (pneumothorax of the right side, pylethrombosis, early cirrhosis of the liver) in which liver dysfunction is absent or too limited to account for the finding. It could also explain the daily fluctuations and the postprandial increase of physiologic urobilinuria.

The findings presented in this paper might have possible diagnostic and therapeutic applications. That the sodium nicotinate technique could be used as a test of liver function is obvious. The technique, producing a prompt and conspicuous rise of the serum indirect reacting bilirubin, tests the ability of the hepatic cell to dispose of a sudden and large load of bilirubin and evaluates both the bile-forming and the bile-excreting functions of the liver. Compared with the tolerance tests of exogenous bilirubin or with other techniques in which a rise of the serum bilirubin level is determined indirectly by the injection of different substances (epinephrine, biliary salts, desoxycorticosterone acetate) or other methods (ingestion of fats and carbohydrates, diathermy and roentgen irradiation of the hepatic and splenic areas), the sodium nicotinate test has the advantage of causing a prolonged and conspicuous hyperbilirubinemia with a technique both simple and safe. Unfortunately, studies on seventy-eight patients presenting various liver disorders show the pattern of the hyperbilirubinemic curve after intravenous injection of sodium nicotinate to present no definite correlation with the anatomic or clinical diagnosis of the case, the total hepatic function evaluated with a "profile" of liver function tests, or the results of any other individual test. This finding is not attributable to an intrinsic defect of the sodium nicotinate technique, but to the fact that bilirubin excretion is widely dissociated from other functions of the liver: it may remain normal even in cases of serious cellular damage, or be involved early in rather benign conditions. The results of the test, therefore, cannot be used as an indication of the total function of the liver nor considered as a means of differential diagnosis between obstructive and hepatocellular jaundice, since a diag-

nostically typical curve (flat type) is obtained only in cases of complete obstruction of the biliary tract. The sodium nicotinate technique remains mainly a test of the bilirubin-excreting function of the liver. Under certain conditions, it may also be useful in the prognostic evaluation of jaundice. For example, in cases of infectious hepatitis still with evident jaundice the test may give a normal result, indicating the restoration of a normal excretion of bilirubin and forecasting the regression of the icterus at an early date. In other cases jaundice may be due rather to sluggishness than insufficiency of the bilirubin-excreting function, as in icteric malaria. The sodium nicotinate curve will then show a normal pattern.

Sufficient evidence has been given to show that sodium nicotinate, when administered intravenously, exercises a powerful cholagogic and choleretic action. This effect has been proved by means of duodenal intubation in normal subjects and in a patient with surgical biliary fistula. Experiments utilizing the same techniques have also shown that intravenous nicotinamide or sodium nicotinate or nicotinamide administered by mouth in therapeutic doses also possess the same action, although slightly less pronounced. Although these findings suggest a possible therapeutic use of both substances in jaundice which has not yet reached the irreversible stage, much more additional evidence is required before these conclusions can be given practical clinical application.

This communication should be considered of a preliminary character and the results presented here certainly do not exhaust the possibilities of experimental and clinical investigation offered by the sodium nicotinate test. It is unfortunate that the related observations cannot be completed and integrated with animal experimentation because no hyperbilirubinemia can be determined in dogs and rabbits with the technique. However, further work in human beings is contemplated, employing this test, to obtain more information on the normal and pathologic metabolism of biliary pigments.

SUMMARY AND CONCLUSIONS

1. The intravenous injection of 30 mg. of sodium nicotinate in normal subjects is followed by: (a) a rise of the serum level of indirect reacting bilirubin, which reaches a maximum after about ninety minutes, then slowly returns to the initial value within six to eight hours; (b) almost immediate stimulation of the secretion and excretion of bile (bilirubin); (c) increased elimination of urobilin, which reaches a maximum two to three hours after the injection and returns to normal values in about twenty-four hours.

2. In pathologic conditions, two main modifications of the hyperbilirubinemic effect of sodium nicotinate are observed: (a) a failure of the bilirubin level to return to normal values within the experimental period of eight hours; (b) the appearance in circulation of bilirubin of the direct reacting type. Their various combinations characterize different types of curves obtained in patients with liver disorders. These curves are not consistently related to the anatomic or clinical condition of the patient. Therefore, the hyperbilirubinemic effect cannot be utilized as a test of liver function or for the differentiation between hepatogenous and obstructive jaundice.

3. The interpretation of the ascending part of the hyperbilirubinemic curve is complex; experimental evidence suggests that the rise in level of the indirect reacting bilirubin in serum might be due to increased reabsorption of mesobilirubin from the intestine. The descending part is in direct relationship to the bile-excreting function of the liver.

4. The increased elimination of urobilin is very likely due to the increased reabsorption of urobilinogen, because of the vasodilatory, cholagogic and choleretic effects of sodium nicotinate. The finding proves that large quantities of the chromogen may appear in the urine even when no liver damage or hyperhemolysis can be demonstrated.

5. Both sodium nicotinate and nicotinamide, when given intravenously or orally, exert a powerful choleretic and cholagogic effect.

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CHOLESTEROL-DESOXYCHOLIC ACID: A STABLE ANTIGEN FOR USE IN A FLOCCULATION TEST FOR LIVER DYSFUNCTION

I. Comparison With the Hanger Cephalin-Cholesterol Test

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SINCE the introduction of the Hanger test,[1, 2] it has found many proponents.[3-12] I had an opportunity to employ this test as well as other liver function studies in a large series of cases in military personnel.

There were a few pitfalls associated with the cephalin-cholesterol flocculation test as it was first performed by me. Bacterial contamination was found to give rise to false positives, especially in an overheated room. Furthermore, the reaction was photosensitive.[13] The latter was reported also by Neefe and Reinhold.[11] To avoid bacterial contamination, sulfanilamide was dissolved in the 0.85 per cent saline in a concentration of 1:1,000, and later, a 1:10,000 solution of Merthiolate was employed. With both of these agents there was found no interference with the sensitivity of the reaction. To avoid the false positives observed with exposure to light, especially direct sunlight, the tubes were kept in a dark cupboard.

It was observed early that there was a considerable disparity of results obtained in the Hanger tests when parallel tests were set up with two commercial antigens, Wilson and Difco. The former seemed to be much less sensitive. It appeared likely that such variation between the two antigens may have been due to differences in the cephalin. Previously, Mateer and co-workers had indicated that the state of the cephalin affected the sensitivity of the test.[6, 14] Later, Lippincott and associates[15] reported a discrepancy of results obtained in patients with vivax malaria, employing the Difco and Wilson antigens. These authors also found the Wilson antigens to be less sensitive.

Kabat, Hanger, Moore, and Landow[16] showed that false positive tests were obtained with known negative sera, when the sera was inactivated for thirty minutes at 56° C. This observation also was made by me in using similar heat-inactivated sera.

There is no attempt made in this paper to detract from the value of the Hanger reaction as a liver function test; in fact, adequate confirmation of the reliability of the test is presented herein. This investigation was undertaken because of the following: (1) the instability of the cephalin in the preparation of the antigen, (2) the difficulty in preparing the antigen or the expense in purchasing commercial preparations of the antigen, and (3) the lack of availability

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of the antigen or the components for its preparation in overseas hospital installations. An attempt was therefore made to prepare a similar antigen from readily available compounds, but one that would be stable. Cephalin is an unstable phospholipid, which readily undergoes oxidation. It appeared likely that the chief function of this substance in the preparation of the Hanger antigen was to render the antigen more soluble in the aqueous media employed in the flocculation test. This fact was also suggested by Maclagan,[17] who, during the development of the thymol turbidity test, suggested that the turbidity obtained with the sera of patients manifesting parenchymatous liver disease was due primarily to phenol and its substitution products. He showed a definite relationship between the molecular weight, solubility, and the precipitating power. He indicated that higher homologues such as cholesterol failed to give positive results because of their lack of solubility.

Consequently, I sought a stable substance of known chemical composition that would increase the solubility of cholesterol. Verzár and Kúthy[18, 19] demonstrated that free bile acids have the ability to keep fatty acids and other acholic components in aqueous solution. This appeared to be accomplished by the formation of coordination compounds and was confined to the alkaline side of the neutral point. These authors also showed that lecithin and other substances exert an influence of their own on solubility in a mixture and may supplement the effect of the bile acids. The enhanced solubility of cholesterol in bile and bile acids solutions was mentioned as early as 1868 by Kuehne.[20] Finally, diffusion experiments by Schoenheimer and Hrdina[21] showed that cholesterol was more strongly associated with desoxycholic acid than any other bile acid.

Hence, desoxycholic acid was selected as the compound of choice, to replace cephalin in the Hanger antigen. Desoxycholic acid has been used in the preparation of a variety of coordination compounds, including the phenanthrene hydrocarbons. It has the property of forming loose compounds with sterids, thereby broadening the solubility range of the combined substance. Initially a coordination compound of cholesterol-desoxycholate was prepared by fusing one molecular weight each of cholesterol and desoxycholic acid with the aid of heat. A cholesterol-desoxycholate was also prepared by the method of Downie, Stent, and White[22] by crystallization from petroleum ether. Subsequently a simpler preparation was devised, which eliminated the necessity for the formation of this coordination compound entirely.

METHOD

Preparation of the Stock Antigen.—1.2 Gm. of chemically pure desoxycholic acid were dissolved in 16 c.c. of absolute alcohol with the aid of gentle heating; then 0.6 Gm. of chemically pure cholesterol was dissolved in this solution of desoxycholic acid. This yields an alcoholic solution of cholesterol which was of the same concentration as that of Hanger. This stock antigen was stored in a tightly stoppered bottle in the refrigerator.

Preparation of the Cholesterol-Desoxycholic Acid Emulsion.—The working antigen was prepared by placing 30 c.c. of N/60 NaOH in a small beaker and heating to about 70° C. The flame was removed and 1 c.c. of stock antigen was added, a drop at a time, with constant stirring. The mixture was then slowly brought just to the boiling point with constant stirring. The resultant emulsion was then allowed to cool to room temperature and transferred to two 15 c.c. centrifuge tubes. These were centrifuged at 1,500 r.p.m. for five

minutes to remove any coarse particles. The supernatant milky emulsion was then poured off and stored in a stoppered test tube. This emulsion keeps for days in the refrigerator.

Procedure.—To 4 c.c. of phosphate buffer of pH 7.8 in a small test tube or 15 c.c. centrifuge tube such as Hanger employed, was added 0.2 c.c. of serum. The contents were then mixed by inverting several times. Then 1 c.c. of antigen emulsion was added and the contents were vigorously shaken, stoppered, and allowed to stand. The tubes were examined at twelve, twenty-four, and forty-eight hours for flocculation and were reported as negative, +, ++, +++, ++++, as in the Hanger test. Two control tubes were prepared at the same time. One contained known negative serum and the other contained just the buffer and emulsion in the same proportions as in the test. Flocculation of ++ or more in forty-eight hours was considered positive.

Parallel Hanger tests were performed in all sera tested. Simultaneous thymol turbidity, icterus index, serum bilirubin, albumin/globulin ratio, cholesterol, total and esters, alkaline phosphatase, and, in many instances, bromsulfalein, heterophile antibody, and urine urobilinogen were also carried out. These latter will be reported in a subsequent paper.[26]

There were 600 tests performed on 485 individuals. The type of cases utilized are depicted in Table I.

TABLE I. CASES STUDIED

DIAGNOSIS	NUMBER OF PATIENTS	NUMBER OF TESTS
Infectious hepatitis	142	174
Obstructive jaundice	14	22
Cirrhosis of liver	5	12
Cholecystitis	8	8
Infectious mononucleosis	38	66
Vivax malaria	82	116
Miscellaneous	28	34
Normal	168	168
Total	485	600

Where possible, the sera of these patients were tested during the various phases of the disease, i.e., at the onset, during subsidence, during recrudescence, and following clinical recovery. The diagnoses in the various cases were based on appropriate laboratory studies and clinical evaluation.*

RESULTS

The results obtained in the individual cases will be published subsequently together with the other laboratory data mentioned previously. A summary of the data is herein presented.

The results obtained in the sera of patients in various stages of infectious hepatitis are shown in Table II.

TABLE II. FLOCCULATION TESTS IN CASES OF INFECTIOUS HEPATITIS

NUMBER OF CASES	NUMBER OF TESTS	% HANGER POS.	% C.D.F.* POS.	CLINICAL STAGE
56	76	96	97	Active (acute)
64	73	92	95	Progressive
22	25	48	11	Recovered†
142	174	78.6	67.6	

*C.D.F., cholesterol-desoxycholic acid flocculation test.

†Icterus index, serum bilirubin normal.

*I am indebted to many medical officers from the 122nd General Hospital, the 135th General Hospital, The Valley Forge General Hospital; especially Lt. Col. I. Pilot, Col. Frank Sinclair, deceased, Lt. Col. E. A. Gall, Capt. Mack Fieber, Lt. Col. R. R. Heffner, Major Jacobson, Dr. H. I. Segal, and Dr. F. R. Schechter for their cooperation, encouragement, and for the clinical diagnosis in the cases studied in this presentation.

The findings in two typical cases are depicted in Table III. These illustrate the close comparison between the Hanger and cholesterol-desoxycholic acid flocculation tests and further indicate alterations in the findings during acute jaundice, progression, subsidence, and recrudescence.

TABLE III. COMPARISON OF HANGER AND C.D.F. TESTS IN TWO PATIENTS WITH INFECTIOUS HEPATITIS

HANGER		C.D.F.		REMARKS
24 HR.	48 HR.	24 HR.	48 HR.	
+4	+4	+4	+4	Acute phase—Patient W. M.
+4	+4	+4	+4	1 wk. later
+4	+4	+4	+4	2 wk. later
+4	+4	+4	+4	3 wk. later
+4	+4	+4	+4	4 wk later, beginning recovery (improvement)
+4	+4	+4	+4	5 wk. later, continued improvement
+4	+4	+4	+4	6 wk. later, continued improvement
+3	+4	+4	+4	7 wk. later, continued improvement
+2	+3	+1	+3	8 wk. later, continued improvement
+1	+2	+1	+3	9 wk. later, continued improvement
0	±	0	+1	11 wk. later, complete clinical recovery
+4	+4	+4	+4	35 days after onset of jaundice—Patient I. G.
+4	+4	+4	+4	45 days after onset of jaundice
+2	+4	+1	+3	58 days after onset of jaundice, clinical improvement
0	0	+1	+2	75 days after onset of jaundice, no jaundice, marked improvement. Icterus index = 9 units
0	±	+1	+3	82 days after onset of jaundice, relapse, icterus index = 15 units
+4	+4	+4	+4	105 days after onset of jaundice, relapse, icterus index = 20 units

In Patient W. M. there was close agreement between the two tests throughout the clinical course. However, in Patient I. G., although the Hanger test became negative at the time of apparent recovery when there was no jaundice, the cholesterol-desoxycholic acid flocculation test continued to show a plus two flocculation forty-eight hours later. Both tests again became positive during the period of recrudescence. No explanation was attempted to account for this discrepancy.

This series of 142 cases will be published in detail in a subsequent paper together with other pertinent laboratory studies.

All of the other types of cases studied were collected together for the presentation in Table IV and constituted 343 patients, which included 168 normal controls.

The results obtained with both the Hanger and cholesterol-desoxycholic acid flocculation tests agreed very closely. In all cases of early extrahepatic biliary obstruction, both tests were uniformly negative. Only one of five cases of cirrhosis of the liver gave a positive flocculation test by both methods. Two cases of homologous serum jaundice were positive by both tests. One case of hemolytic jaundice manifested positive flocculation with the two antigens. In a series of thirty-eight cases of infectious mononucleosis, twenty-six patients gave positive Hanger tests and in twenty-eight the cholesterol-desoxycholic acid

TABLE IV. COMPARATIVE VALUES IN MISCELLANEOUS HOSPITAL CASES

NUMBER OF CASES	NUMBER OF TESTS	% HANGER POS.	% C.D.F. POS.	REMARKS
14	22	0	0	Early extrahepatic biliary obstruction
5	12	20	20	Cirrhosis of liver
2	6	100	100	Homologous serum jaundice
8	8	0	0	Acute cholecystitis
38	66	68	74	Infectious mononucleosis
82	116	75	75	Vivax malaria, during paroxysm
1	2	100	100	Hemolytic jaundice
2	2	0	0	Amebiasis with jaundice
1	2	0	0	Sickle cell anemia
2	2	0	0	Nutritional deficiencies
8	8	75	75	Atypical viral pneumonia
1	1	0	0	Pulmonary tuberculosis
9	9	0	0	Upper respiratory infection
1	1	0	0	Peptic ulcer
1	1	0	0	Acute hemorrhagic pancreatitis
168	168	0	0	Normal
343	426			

flocculation test was positive. Of eight cases of atypical pneumonia, 75 per cent gave positive flocculation by both methods; of eighty-two cases of malaria due to *Plasmodium vivax*, 75 per cent yielded positive flocculation with both antigens, when the sera was tested during the paroxysm. In most, the tests became negative with the termination of the fever episode.

Flocculation tests were carried out on a series of six rabbits. The results are summarized in Table V. These tests were performed because of reports[23, 24, 25] that the Hanger test was positive in these animals and the colloidal gold and thymol turbidity tests were negative. Recant, Chargaff, and Hanger[23] also demonstrated negative thymol turbidities when physiologic salt solution was substituted for distilled water in the preparation of the buffer with sera that previously yielded positive tests. When physiologic saline was substituted for the phosphate buffer in the cholesterol-desoxycholic acid flocculation test, the positivity of the tests was not altered.

TABLE V. FLOCCULATION TEST IN RABBITS WITH C.D.F. TEST

RABBIT	24 HR.	48 HR.
1	+	++++
2	±	++
3	0	++
4	++	++++
5	+	+++
6	0	+

DISCUSSION

Desoxycholic acid was selected to replace the cephalin in the Hanger antigen. The desoxycholic acid was chosen because it increased the solubility of cholesterol in aqueous media, was stable, of known chemical purity, economical, and readily available. Initially, a coordination compound of cholesterol-desoxycholate was employed in the preparation of the stock antigen. Later, it was determined that a mixture of desoxycholic acid and cholesterol in absolute alcohol would suffice. In making the working antigen-emulsion, N/60 sodium

hydroxide was employed instead of distilled water, because of the greater solubility of the ingredients on the alkaline side of the neutral point. As few departures from the actual technique of Hanger were introduced as possible, in order to draw closer comparisons.

In all, 600 tests were performed on 485 patients, using both the Hanger and cholesterol-desoxycholic acid antigens and the same samples of sera. There was an over-all agreement of 94 per cent between the two tests, with the cholesterol-desoxycholic acid flocculation test slightly more sensitive. Although no comparisons of the reactions in individual cases, with the exception of Table III, were tabulated in this paper, the data indicates close agreement.

The cholesterol-desoxycholic acid flocculation test appeared to compare favorably with the Hanger test in early diagnosis of hepatocellular disease, as in infectious hepatitis as well as in the follow-up of these cases during recovery and recrudescence. In one of the cases in Table III, the cholesterol-desoxycholic acid flocculation test was positive in the preicteric period prior to recrudescence, while the Hanger test was negative. The results in early extrahepatic biliary obstruction, cirrhosis of the liver, homologous serum jaundice, and acute cholecystitis show 100 per cent agreement between the Hanger and cholesterol-desoxycholic acid flocculation tests.

Both tests tended to be positive in infectious mononucleosis since in sixty-six tests on thirty-eight patients, 68 per cent of the cases gave positive Hanger and 74 per cent positive cholesterol-desoxycholic acid flocculation tests. These results are in agreement with those of Gall[28] who found a positive cephalin-cholesterol flocculation test in 76 per cent of thirty-four cases of infectious mononucleosis.

In eight cases of atypical pneumonia, 75 per cent showed positive Hanger and cholesterol-desoxycholic acid flocculation tests. Dreyfuss[25] also reported positive cephalin-cholesterol flocculation in three cases of atypical pneumonia.

There was uniform agreement in 116 cholesterol-desoxycholic acid flocculation and Hanger tests in vivax malaria during the paroxysm. These cases were studied[27] at the onset of the chill during the fever bout at frequent intervals. Seventy-five per cent of these cases gave positive flocculation during the paroxysm. These findings have been reported by others.[24, 29, 30, 31] Ten cases of artificial fever gave uniformly negative flocculation tests.[27]

Flocculation tests with cholesterol-desoxycholic acid antigen with the sera of six rabbits revealed results of various positivity. This test was carried out because it was reported that the sera of normal rabbits gave positive flocculation with the Hanger antigen.

Detailed laboratory studies and the results of the Hanger and cholesterol-desoxycholic acid flocculation tests in individual cases have been reserved for a subsequent publication[26] since it was feared that if the data were included herein, the reader would "lose sight of the forest for the trees."

There has been no opportunity to analyze the flocculant in cholesterol-desoxycholic acid flocculation tests electrophoretically and to make a chemical comparison with that obtained in the cephalin-cholesterol flocculation test.

Physically, the flocculant of the cholesterol-desoxycholic acid flocculation test appears to be finer and to pack more firmly. An effort will be made to perform these tests subsequently.

The evidence reported herein would suggest that the Hanger and cholesterol-desoxycholic acid flocculation tests could be employed interchangeably in the diagnosis and follow-up of cases of hepatocellular diseases.

SUMMARY

1. A new antigen is described in which desoxycholic acid was substituted for cephalin in the cephalin-cholesterol flocculation test.

2. There were 600 tests on 475 patients carried out employing the cholesterol-desoxycholic acid (C.D.F.) and the cephalin-cholesterol flocculation test with 94 per cent agreement.

3. The cholesterol-desoxycholic acid flocculation test compared closely with the Hanger test in specificity and sensitivity. The mechanism of action in the two tests appeared to be closely related.

4. The cholesterol-desoxycholic acid flocculation test appeared to be of advantage in regard to stability, ease of preparation, and economy.

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A CRYOGLOBULIN PRESENT IN HIGH CONCENTRATION IN THE PLASMA OF A CASE OF MULTIPLE MYELOMA

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THE first reported case of a reversible cold-precipitation of plasma protein is that of Wintrobe and Buell[1] (1933). This and other cases that have been reported since that time are summarized by Lerner and Watson[2] (1947), who give the relevant bibliographies. In their paper a new case is described which is particularly valuable because of the completeness of the studies. An extensive physical chemical study of the protein is described in a separate paper by Lerner and Greenberg.[3] All of these cases gave immediate gross precipitation of protein on cooling the plasma and resolution of the precipitate on warming. Because of this property Lerner and Watson have suggested the name "cryoglobulin" for this class of proteins.

Since the publication of the paper of Lerner and Watson, a case with Raynaud's syndrome having a protein of this class in the plasma was reported by Hansen and Faber[4] (1947). Olhagen[5] (1948) has also described three cases that merit special mention. All three gave spontaneous crystalline precipitates, although the crystal structure was not the same in any two cases. In Case I (diagnosis, myeloma; total plasma protein, 10.1 Gm. per cent), the content of spontaneously precipitating protein was 1.4 Gm. per cent of plasma. In spite of this high concentration it did not precipitate immediately on cooling, and after warming to 37° C., redissolved slowly (twenty to thirty minutes). There is a question whether Olhagen's Case II (diagnosis, myeloma; total protein, 13.0 Gm. per cent) should be listed as a cryoglobulin because the precipitation was "on the whole independent of the outer temperature, but slightly faster in the cold." "The spontaneous precipitation of crystalline protein appeared first after 24 hours" and amounted to 0.8 Gm. per cent of plasma. The electrophoretic pattern of this plasma showed 75.6 per cent of the total area in the gamma globulin peak. This is reminiscent of the case of Holmberg[6] in which precipitation could be prevented by anaerobic collection. The stimulus to precipitation seemed to be the loss of CO_2 on exposure to air. Olhagen did not find anaerobic collection a factor in his Case II and does not suggest a cause for the protein precipitation. Olhagen's Case III (probably "a myeloma diagnosis in spite of the negative marrow and x-ray findings") was complicated by renal insufficiency and high blood nonprotein nitrogen. There was no hyperproteinemia (total protein, 7.4 Gm. per cent). The spontaneously crystallizing protein amounted to 0.6 Gm. per cent of plasma; it precipitated immediately on cooling and redissolved promptly on warming to 37° C. A urinary protein was electrophoretically identical with the cryoglobulin of the plasma.

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Wertheimer and Stein[7] were the first to suggest a classification of "cold fraction" plasma proteins. They made a systematic search for such proteins following their discovery of a "cold fraction" in the plasmas of dogs infected with kala-azar. "Cold fractions" were classified by them as Grades 1, 2, 3, 4, and 5. Grade 1 was defined as including those plasmas showing "turbidity marked but no precipitate" after standing twenty-four hours in the refrigerator at 7 to 11° C.; Grades 2 and 3 were intermediate and Grade 4 included plasmas showing a "marked precipitate easily estimable by quantitative methods." Grade 5 was reserved for reversible cold fractions of a nonprotein nature, usually cholesterol, of which these authors found a considerable number. Investigators are warned against mistaking Grade 5 cold fractions for cold-sensitive proteins. Five cases of kala-azar in human beings were investigated, two of which showed Grade 4 cold fractions while the other three showed Grade 3 precipitations. This indicates that large quantities of cold-sensitive plasma proteins may be a common occurrence in this disease to be found for the searching. Eight cases of endocarditis lenta all showed cold fractions of Grades 1 to 3, with one showing Grade 4. Grades 1 to 3 were found in four cases of nephrosis. In the twenty-seven cases studied, Grade 5 precipitates (nonprotein reversible cold fractions) were found in six.

A more extensive and more systematic search for cold-sensitive plasma proteins was reported by Lerner, Barnum, and Watson[8] (1947). These authors examined the serums from one hundred twenty-one patients with a variety of pathologic conditions and from forty controls. Their work is the basis for a very practical classification of cryoglobulins according to the time of appearance on standing at 5° C. and according to the amount of precipitate formed. Grade 1 includes plasmas containing a trace to 6 mg. per cent of cryoglobulin appearing between twenty-four hours and one week; Grade 2 includes plasmas containing 6 to 25 mg. per cent appearing in twenty-four hours; Grade 3 includes plasmas containing more than 25 mg. per cent of cryoglobulin appearing immediately on cooling. In one hundred twenty-one cases examined they found twelve cases of Grade 2 and eighteen cases of Grade 1 cryoglobulinemia. None were found among the forty controls. No case of Grade 3 cryoglobulinemia was found because of the systematic search; the one case listed was the first seen by these authors and was apparently the stimulus for the later work. The behavior of lipid precipitates was found to be "entirely different from the cryoglobulins." It should be emphasized that the Grade 3 cryoglobulinemia of these authors, the grade including the heaviest precipitation, corresponds to Grade 4 of Wertheimer and Stein.[7] In our search of the literature, we have found, including our own, fifteen cases of cryoglobulinemia that would be classified as Grade 3 according to the classification of Lerner, Barnum, and Watson.[8]

CASE REPORT

In the present paper we are reporting some properties of the plasma and of a cryoglobulin separated from the plasma of a person (Patient O. O.) suffering from multiple myeloma. The patient, a white woman, 55 years of age, entered Colorado General Hospital

Fig. 1.—X-ray of left shoulder showing extensive destruction of head of left humerus and osteolytic process in the left scapula and clavicle.

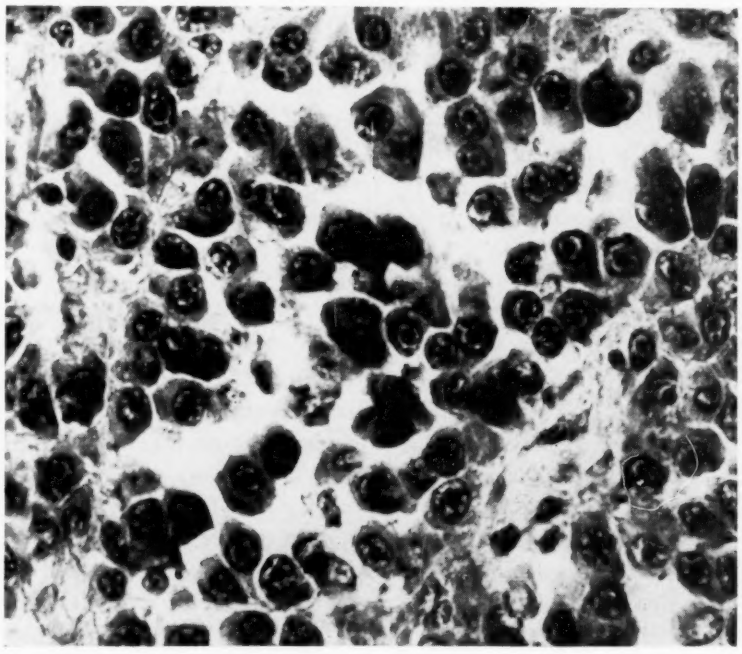

Fig. 2.—Plasma cell myeloma of left humerus. Note prominent nuclei, often eccentric, sometimes double, with heavy, coarse, marginated chromatin and large conspicuous nucleoli. Hematoxylin and eosin stain. (×600.)

on Aug. 7, 1945. For the past seven years she had suffered from severe sensitivity to cold. Death occurred the following September 9. Autopsy revealed plasma cell myeloma involving the bone marrow, chiefly of the left humerus, scapula, and clavicle. The bone of the head and neck of the humerus was extensively destroyed by massive proliferation of cells with the morphology of plasma cells, chiefly in the plasmoblast stage. (Figs. 1 and 2.)*

RESULTS

Properties of the Plasma.—When the blood of Patient O. O. was first drawn it was found to solidify quickly in the needle and syringe even though large amounts of anticoagulant were used. However, when the syringe and needle were warmed before use and the sample of drawn blood was kept warm, it remained liquid indefinitely with no more than the usual additions of anticoagulant. When heparinized blood was kept at 37 to 40° C. throughout the process of centrifugation it separated into three layers: an upper layer of apparently normal plasma; a middle, clear, almost water-white layer; and a lower layer of cells. On cooling, the upper layer remained liquid. The middle layer, however, congealed to a pearly white translucent solid. In a sample of 7.5 ml. of blood, the volumes of the three layers were: upper, 3.0 ml.; middle, 2.8 ml.; and lower, 1.7 milliliters. Thus the middle layer of cold-sensitive material accounted for 37 per cent of the total volume of the blood. When the middle layer solidified on cooling, the top layer could be poured off and, with distilled water, could be washed off completely from the surface of the solid middle portion. On warming, the middle layer again liquefied and could be pipetted from the surface of the cells. This separation was accomplished on five different samples drawn on five different days. Following a whole blood transfusion the separation into three layers never again occurred though the whole blood or plasma solidified on cooling as before. After dilution with distilled water the cryoglobulin of these latter samples separated on cooling first as a thin gel which then quickly broke up as a flocculent precipitate and could be purified by resolution in warm water from which it again precipitated on cooling. Solidification on cooling to about 32° C. and liquefaction on rewarming to 38° C. was repeated on the same sample of whole blood fifteen times without any apparent changes in its properties.

Fractional plasma protein analyses were carried out by means of a micromodification[10] of the method of Howe. These and other analytic data are collected in Table II. The urine was free from protein at all times.

In dilute solution the cryoglobulin was precipitated solely in the globulin fraction. This was not true in high concentrations and it is probable that the high "fibrinogen" fraction on Aug. 28, 1945, contained a large amount of cryoglobulin.

The viscosity of the plasma was measured at different temperatures with the Ostwald viscosimeter. Exactly 3 ml. of the plasma were used in the instrument which was then placed in a water bath at 45° C. and allowed to stand for thirty minutes to come to thermal equilibrium. After several determinations of viscosity were made at this temperature, the bath was cooled quickly with crushed ice and a new temperature level established. By repeating this procedure, the data for viscosity changes with temperature were obtained.

*The pathologic aspects of the case have been reported.[9]

In Fig. 3 the curve for the plasma of Patient O. O. is compared with the average curve for three normal plasmas measured in this laboratory and the average curve for three normal plasmas and the curve for one hyperproteinemic plasma (9.2 Gm. per cent) reported by Jochims.[11] The viscosity of the plasma of Patient O. O. is more than five times that of the average normal plasma at 38° C. and rapidly increases to "infinity" at 32° C. If the viscosity of normal

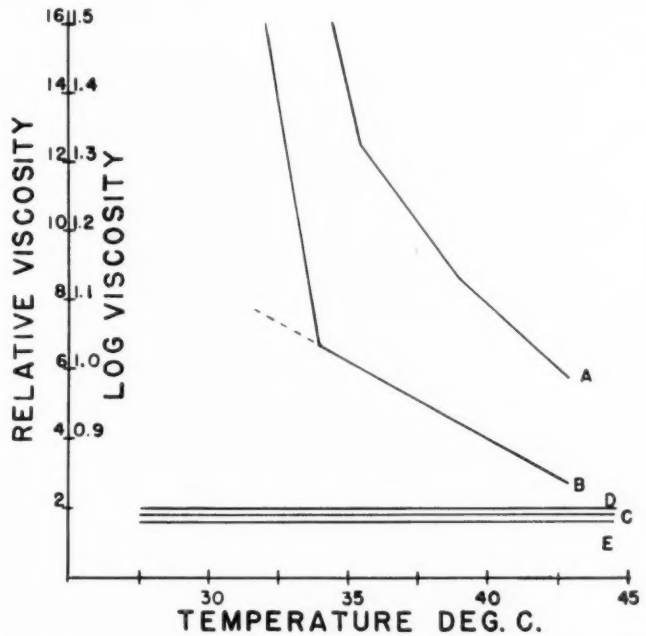

Fig. 3.—The changes of viscosity with temperature of the plasma of Patient O. O. *A*, A case of hyperproteinemia reported by Jochims[11]; *D*, the average of three normals reported by Jochims (*C*); and *E*, the average of three normals examined in this laboratory. Curve *B* is the log of the viscosity of the plasma of Patient O. O., showing a break at about 34° C.

plasma is plotted against the logarithm of the temperature, a continuous straight line is obtained. When the viscosity of the plasma of Patient O. O. is so plotted, two straight lines are obtained. The position of the break in the semilogarithmic curve suggests that the change in state of the cryoprotein on cooling begins when the temperature reaches 34° C.

Chemistry of the Cryoglobulin.—A large sample of whole plasma was diluted and cooled in the icebox for several hours. It was then centrifuged with the centrifuge tubes packed in ice. The cryoglobulin separated in this way was quickly and completely dissolved when placed in warm (40° C.) distilled water. The solution was again cooled in the icebox to reprecipitate the cryoglobulin. In this way the sample was purified by ten reprecipitations from distilled water. A small sample of the final product was dried to a pure white powder, which gave strong biuret, xanthoproteic, Millon, and Aeree-Rosenheim tests and a negative Molisch test. The bulk of the product was redissolved in warm distilled water and mixed with an equal volume of

95 per cent ethyl alcohol and placed in the icebox. Amorphous material began to precipitate at once and after three days needlelike crystals appeared (Fig. 4). These were at first presumed to be crystals of the cryoglobulin because of the similar crystals found by Wintrobe and Buell[1] and the protein crystals reported in the case of myeloma of von Bonsdorff, Groth, and Packalen.[12] However, the crystals proved to be not protein but cholesterol ester (method of Schoenheimer and Sperry[13]) with no free cholesterol and no

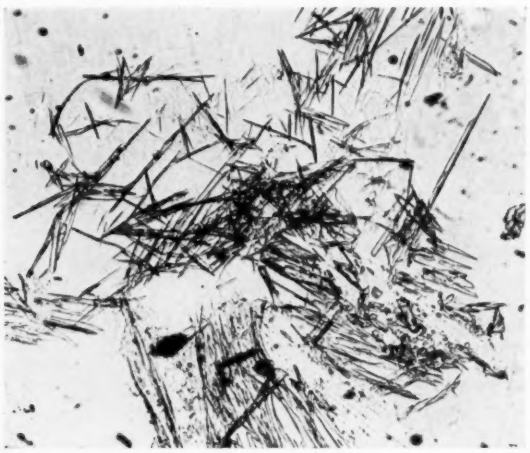

Fig. 4.—Crystals of cholesterol ester separated from purified protein after standing in the cold room three days in 50 per cent ethyl alcohol. (×60.)

nitrogen or phosphorus present. The crystals must represent a "lightly bound" lipid fraction in the protein molecule because although they were not removed by ten resolutions in and precipitations from distilled water, they were removed by treatment in the cold for three days with 50 per cent ethyl alcohol. The quantity of crystals was approximately 2 per cent of the cryoglobulin.

The remaining amorphous protein was dried and subjected to repeated extraction with a 1:2 mixture of ethyl alcohol and ethyl ether. This treatment yielded an additional 0.17 per cent of material which proved to be 20 per cent cholesterol ester and 70 per cent fatty acid. Again no free cholesterol was present and only a trace of lipid phosphorus.

Four samples of the extracted and dried protein were subjected to micro-Kjeldahl analysis[10] and were found to contain 15.2, 15.2, 15.2, and 14.9 per cent nitrogen respectively, with an average of 15.13 per cent. Phosphorus and purines were not present in detectable quantities.

Another sample was assayed for amino acids by microbiologic methods essentially those of Stokes, Gunness, Dreyer, and Caswell[14] and Stokes and Gunness.[15] Nine amino acids were determined with *Streptococcus faecalis* (American Type Culture Collection No. 9790) and the tenth, phenylalanine, with *Lactobacillus delbrueckii* LD 5 (American Type Culture Collection No. 9595).

In Table I these assays are compared with assays, taken from the literature, of some normal plasma protein fractions and with Bence Jones protein. The cryoglobulin shows an amino acid pattern suggesting relationship to the beta or gamma globulins.

TABLE I. AMINO ACID ANALYSES OF CRYOGLOBULIN COMPARED WITH ANALYSES OF NORMAL PLASMA PROTEINS AND BENCE JONES PROTEIN

AMINO ACID	CRYOGLOBULIN (GM. %)	BENCE JONES PROTEIN (GM. %)		ALBUMIN‖ (GM. %)	β-GLOBULIN‖ (GM. %)	γ-GLOBULIN‖ (GM. %)	FIBRINOGEN (GM. %)
Histidine	1.96	1.1*	1.2†	3.5	2.50	2.50	2.8
Arginine	5.56	5.2*	5.0†	6.15	5.64	4.80	7.9
Lysine	7.28	7.0*	6.5†	5.8		6.7	
Tryptophane	1.59	2.5*	2.5†	0.19	2.06	2.86	3.22
Valine	9.60	5.6‡					
Leucine	8.70	6.5‡	10.6§	11.9	8.9	9.32	7.1
Isoleucine	2.13						
Phenylalanine	3.99	4.8‡	1.5§				
Methionine	.90			1.28	1.54	1.06	2.52
Threonine	5.88			5.03	7.26	8.36	6.6

*First case of Calvery and Freyberg.[16]
†Second case of Calvery and Freyberg.[16]
‡Hopkins and Savory[17] (average of two cases).
§Abderhalden and Rostoski.[18]
‖Brand, Kassell, and Saidel.[19]

All of the analyses, the physical properties, and the behavior toward Na_2SO_4 fractionation of this cryoglobulin are compatible with its classification as a pseudoglobulin. It differed from other cryoproteins that have been described in its higher concentration in the plasma and the higher temperature at which it precipitated on cooling. After removal of the cryoglobulin the remaining plasma appeared to be qualitatively normal, though the albumin concentration was low and the globulin and fibrinogen were high (Table II).

TABLE II. PLASMA PROTEIN AND OTHER LABORATORY FINDINGS ON CRYOGLOBULIN-CONTAINING PLASMA OF PATIENT O. O.

	DATE				
	8/11/45	8/24/45			8/28/45
PLASMA	WHOLE PLASMA	WHOLE PLASMA	UPPER LAYER	MIDDLE LAYER	WHOLE PLASMA†
Total protein (Gm. %)	7.2	10.0	7.1	13.1	18.1
Albumin (Gm. %)	2.2	1.8	2.1		2.1
Globulin (Gm. %)	5.0*	6.5	4.5		9.8
Fibrinogen (Gm. %)		1.7	0.5		6.2
N.P.N. (Mg. %)	27	29	37		27
Prothrombin (% normal)	44				
Total cholesterol (Gm. %)	146				122
Serum Ca (Gm. %)	10.4				10.4
Serum P (Gm. %)	4.2				7.1
Alk. phosphatase (Bodansky units)	2.4				2.4
Cold agglutinins	Neg.				
	DATE				
WHOLE BLOOD	8/11	8/20			8/28
Hemoglobin (Gm. %)		5.5			4
Erythrocytes	3,250,000	1,950,000			
Leucocytes	6,700	7,200			
Segmented neutrophils	48	38			
Lymphocytes (%)	50	61			

*Globulin plus fibrinogen.
†A whole blood transfusion was given on 8/26.

DISCUSSION

The cryoglobulin separated from the plasma of Patient O. O. was present in concentrations greater than any so far reported in the literature. It differed from most of the cryoglobulins described in its high solubility in warm distilled water, a property which may have permitted the patient to survive until such high plasma concentrations were attained. Even at body temperature the viscosity of the plasma was greatly increased by the presence of this protein, a fact that had a severely disabling effect upon the patient. The viscosity increased with decreasing temperature until, in terms of the method used, it became "infinite" at 32° C.

Hansen and Faber[4] reported the case of a 7-year-old boy suffering from Raynaud's disease in which no abnormal increase of the viscosity of the plasma was found on cooling, but in which they observed in the plasma "twice, after standing, a beautiful division into two layers. . . . It was a capricious phenomenon that could not be reproduced at will." The abnormal layer amounted to about 10 per cent of the total blood volume and was seen after the plasma stood for several hours. Bing[20] reported a case of multiple myeloma in which the serum was "markedly viscous" and on standing in the refrigerator at 0° C. separated into an upper pale-yellow fluid layer, amounting to four-fifths of the serum, and a lower colorless layer "that froze into a white mass." The lower layer had a total protein content of 17.18 Gm. per cent of which globulin made up 15.82 grams. (See also one case reported by Wallenström.[21]) In the plasma of Patient O. O. the separation into two layers occurred only after centrifugation, amounted to 37 per cent of the total blood volume, and was seen every time warm blood was centrifuged before the patient was given a whole blood transfusion. After the transfusion, though it was looked for frequently, separation of the plasma was never again observed.

The great increase in the globulin content of the plasma of Patient O. O. (Table II, 8/28) reflects the tremendous terminal metabolic activity of the neoplastic cells. We would be inclined to attribute this concentration increase to dehydration if it were not for the nearly constant concentration of albumin and nonprotein nitrogen.

SUMMARY

1. The blood of a patient with multiple myeloma solidified, reversibly, on cooling to 32° C.

2. The plasma, on centrifugalization, separated into two layers, an upper layer of normal appearance and a lower (37 per cent of the whole blood) which was pearly white and proved to be a cryoglobulin.

3. The separation of the plasma into layers did not occur after the patient was given a whole blood transfusion.

4. Changes of the plasma viscosity with changes in temperature are compared with similar measurements on normal plasma and plasma from one other case of hyperproteinemia.

5. Treatment of the purified cryoglobulin with 50 per cent ethyl alcohol in the cold for three days caused the separation of cholesterol ester which crystallized in small needles.

6. Assay of the cryoglobulin showed an amino acid distribution suggestive of beta or gamma globulin.

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IMMUNOCHEMICAL ESTIMATION OF THE RATE OF DISAPPEARANCE OF TRANSFUSED GAMMA GLOBULIN FROM THE BLOOD IN TWO CASES OF HYPOPROTEINEMIA

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SEVERAL cases have been reported in which an unusually low serum gamma globulin was observed electrophoretically.[1-5] In two of these, reported in detail elsewhere,[3, 4] an opportunity was presented to follow by immunochemical methods the disappearance of gamma globulin from the blood after a transfusion of plasma. The quantitative precipitin method has been used by several investigators for the estimation of various serum proteins[6, 7, cf. 8] and more recently a micromodification[9] has been applied to the determination of the gamma globulin and crystallizable albumin in human cerebrospinal fluid.[10] The latter technique was applied in this study and indicated a "half-life" of 3 to 4 days for gamma globulin.

In one of the patients, the ability to form antibodies was tested by subcutaneous injection of blood group A substance[11, 12] and of a mixture of six type-specific pneumococcal polysaccharides.[13, 14, 15] The degree of utilization of dietary amino acids for tissue synthesis was evaluated in the second patient by determining the rate of excretion of N^{15} in the urine after ingestion of a known amount of isotopic glycine.[16]

EXPERIMENTAL

One patient, E. B., was a 31-year-old woman. Her case history has been presented by Homburger and Petermann[3] in connection with a detailed description of a new syndrome, familial idiopathic hypoproteinemia. Despite a constant high protein diet, the gamma globulin level remained low. No evidence of inefficient utilization of dietary protein could be found. Proteinuria was absent. The rate of synthesis of serum protein was found to be normal.[3] The general clinical picture was negative.

A small blood sample (E. $B._x$) was taken before the intravenous injection of 975 ml. of normal donor plasma. Blood samples were then removed two hours later and after one, three, five, and nine days.

Determination of the gamma globulin in the transfused plasma and in the serum samples was carried out as previously described[10] except for the addition of appropriate dilutions of the serum samples to 1.0 ml. portions of calibrated rabbit antiserum to purified and electrophoretically homogeneous human gamma globulin ($gamma_2$ globulin of Deutsch and coworkers[17]) and measuring the quantities of specifically precipitable nitrogen. The equivalent amounts of gamma globulin are found by interpolation on the calibration curve. Dilutions of the serum samples of 1:50 to 1:200 were required to ensure that all analyses were carried out in the region of antibody excess (as is necessary for the immunochemical method to be valid). The results are listed in Table I.

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TABLE I. DISAPPEARANCE OF GAMMA GLOBULIN ON INJECTION OF NORMAL PLASMA

BLOOD SAMPLE	NUMBER OF DAYS AFTER TRANSFUSION	GAMMA GLOBULIN PER ML. (MG.)	TOTAL PROTEIN PER 100 ML. (GM.)
		Patient E. B.	
Donor plasma		12.4	6.15
E. $B._x$		3.4	5.31
E. $B._1$	(2 hr.)	6.6	5.49
E. $B._2$	1	6.1	6.01
E. $B._3$	3	5.2	5.83
E. $B._4$	5	4.5	5.27
E. $B._5$	9	3.1	5.13
		Patient B. V.	
Donor plasma		11.5	5.84
B. $V._x$		3.1	3.58
B. $V._1$	(2 hr.)	5.6	4.36
B. $V._2$	1	4.6	3.90
B. $V._3$	3	4.1	3.81
B. $V._4$	5	3.4	
B. $V._5$	9	3.6	3.56
B. $V._6$	14	3.0	3.28

Subscripts: x, Preinjection level. 1, Level in blood sample taken two hours after injection of plasma.

To determine the antibody response on injection of hog blood group A substance, the patient (E. B.) received subcutaneously on two occasions two days apart a total of 2 mg. of a purified hog stomach blood group A preparation (preparation 2A[11]). Examination of a blood sample obtained ten days later revealed no significant change in antibody level.

The patient also received an 0.8 ml. injection of a mixture of pneumococcal I, II, III, V, VII, VIII type-specific polysaccharides which also contained some group-specific "C" substance.*† Analyses were performed[14]‡ on bleedings taken just before and three weeks after the immunization. The results are listed in Table II.

TABLE II. ANTIBODY LEVEL IN E. B. AFTER INTRACUTANEOUS INJECTION OF SPECIFIC POLYSACCHARIDES OF TYPES I, II, III, V, VII, AND VIII

	ANTIBODY N PER 4 ML. SERUM						
	ANTIBODIES TO						
	C	I	II	III	V	VII	VIII
Before	0.003 μg	0 μg	0 μg	0 μg	0 μg	0 μg	0 μg
After immunization	0.003 μg	0.001 μg	0.005 μg	0 μg	0 μg	0.018 μg	0.004 μg

The second patient, B. V., a 33-year-old female mechanic, showed a generalized nonspecific lymph node enlargement in 1937 which was radiosensitive and disappeared on treatment in August, 1938. The albuminuria noticed in 1938 did not reappear. With the exception of edema of the lower extremities, the clinical picture was negative. An intake of 90 Gm. of protein per day was required to maintain nitrogen balance. For fifteen days before and for the duration of the experiment (Jan. 9 to 23, 1948) the daily protein intake was about 150 Gm. per day. A detailed case history will be given elsewhere.[4] The patient died on March 13, 1948, from a brain hemorrhage, and a giant follicular lymphoma was found on autopsy.

One-half liter of normal plasma was injected intravenously after a blood specimen (B. $V._x$) had been taken. Additional blood specimens were removed two hours, one, three, five, nine, and fourteen days later for total protein and gamma globulin analyses. As in the case of E. B., serum samples prepared from the bleedings were diluted appropriately; the gamma globulin determinations are listed in Table I. Duplicate analyses were performed throughout.

*Manufactured by E. R. Squibb and Co., New Brunswick, N. J.

†The vaccine was obtained through the kindness of Professor Michael Heidelberger.

‡By Mrs. Marie DiLapi in Dr. Heidelberger's laboratory.

To obtain information concerning the efficiency of utilization of dietary glycine,[16] B. V. received orally 540 mg. of glycine[18]* containing 32 per cent excess N^{15}. Total daily urine collections were made for three days. Isotope determinations† indicated that 42, 52, and 60 per cent of the dietary isotope had been eliminated twenty-four, forty-eight, and seventy-two hours after ingestion.

RESULTS

From Table I it may be seen that injection of the plasma caused almost a doubling of the gamma globulin concentration in the blood serum of both subjects, although there was a very much smaller increase in total serum protein. In both instances, it required about nine days for the gamma globulin to return to the preinjection level, and about three to four days for the initial increase in gamma globulin to be reduced by one-half, indicating a "half-life" for the injected gamma globulin of about 3 to 4 days.

As for the response of E. B. to injection of pneumococcal specific polysaccharides, the data in Table II reveal no measurable antibody to types III and V and extremely little to type I. The greatest response was to type VII. The values for types II and VIII are probably significant.

DISCUSSION

Although many electrophoretic studies on plasma proteins have been carried out, only very few instances of low gamma globulin have been recorded. Perhaps the first record is that by Forbes in 1944[1] of a hypoproteinemic woman. The gamma globulin studied electrophoretically was unaffected by ingestion or intravenous injection of Amigen. Following a plasma transfusion, this patient eliminated most of the plasma protein she had received intravenously in eight days. Krebs[2] described a 15-year-old girl suffering from malnutrition who showed a general hypoproteinemia with an electrophoretically determined gamma globulin level of 0.15 Gm. per 100 ml. plasma. This returned to about normal (0.65) on dietotherapy. Two other cases of hypogammaglobulinemia, in children, have been cited by Stern.[5]

In the present study, an attempt has been made to consider the possible metabolic basis for the low gamma globulin levels. It has been seen that a decreased gamma globulin due to low protein intake may be restored to normal when the proper protein intake is maintained.[2] This explanation obviously does not apply to the two patients studied, for they were kept on protein intake levels far in excess of that required for nitrogen balance without affecting their serum gamma globulin.

The present study showed that on transfusion of an amount of plasma sufficient to double the circulating serum gamma globulin, this component returned to its original level at a rate indicating a "half-life" of 3 to 4 days. Unfortunately, the procedure does not lend itself to a comparison in normal subjects since it would not be possible to raise the gamma globulin level significantly by transfusing normal plasma into a normal subject. It therefore becomes necessary to compare the "half-life" of gamma globulin

*Obtained through the kindness of Dr. David Rittenberg.
†Carried out by Mr. I. Sucher in Dr. Rittenberg's laboratory.

found by this method with that found by other methods. A "half-life" of about two weeks has been found for human serum globulin by isotope studies.[13, cf. footnote, p. 317]

The specificity of the immunochemical method, as applied to the estimation of gamma globulin, has been considered previously.[10] Deutsch and coworkers[19] have found extensive cross-reactions with human gamma$_1$ and gamma$_2$ globulin. Very recently it has been reported[20] that several preparations of human gamma$_2$ globulins react alike with rabbit antigamma$_2$ serum. Since immunochemical determinations of serum gamma globulin were consistently higher than the electrophoretic values, Jager, Smith, Nickerson and Brown[20] inferred that other serum proteins (probably globulins) cross-reacted with the antiserum. The individual values obtained in this investigation therefore probably represent all constituents reactive with antibody to gamma$_2$ globulin. There is, however, general agreement between the values reported here and those for gamma globulin obtained on the same serum samples (from E. B.) by electrophoresis,[3] from which the same "half-life" can be calculated.

To determine antibody production, one of the patients (E. B.) received injections of the blood group substance[11, 12] and several type-specific pneumococcal polysaccharides.[13, 14, 15] There was no response to the A substance and very little or no anti-SI, -SIII, and -SV formed. The patient did, however, form antibodies against types II, VIII, and VII, although the antibody levels reached were not striking. These data, while below average, do not differ sufficiently to be abnormally low. It might be of interest in this connection to compare these findings with the report[21] that rabbits showed a lower capacity to produce agglutinins against *Eberthella typhosa* when made hypoproteinemic by low protein diets or plasmapheresis.

When the high protein diet of the second patient (B. V.) is taken into account, the rate of excretion of isotope in the urine after ingestion of a single small dose of isotopic glycine[16] suggests a rather normal utilization for protein synthesis.

SUMMARY

1. The rate of elimination of transfused gamma globulin from the blood stream of two patients with idiopathic hypoproteinemia was followed immunochemically by the quantitative precipitin method.

2. The "half-life" of transfused gamma globulin in these patients was found to be three to four days.

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SENSITIZATIONS TO THE FACTOR Rh IN NEGROES

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THE literature on Rh sensitizations in Negroid races is exceedingly sparse, and with the exception of one report by Wiener and Wexler[1] no systematic study of this problem has come to our attention. In that report, three cases of erythroblastosis fetalis in Negroes are described, none due to Rh and two due to apparent sensitizations against the factors A or B. The inference is made that in this group of people there might be a better placental barrier or there may exist a lower sensitivity to the antigen Rh. In a subsequent personal communication to one of us (R. K. W.) it was pointed out that the intentions of that paper were actually to report three unusual cases and to arouse interest in the subject matter and not to make any generalized statements concerning Rh sensitizations in Negroes.

It is the purpose of this paper to describe our experience with isoimmunization in Negroes taken at random from the obstetric clinic of the Medical College of Virginia.

MATERIALS AND METHODS

During the past two years 3,115 consecutive obstetric clinic patients were examined for blood groups, Rh factor, and presence of isoantibodies. Those found to be negative with anti-D (Rh_0) serum were checked at approximately monthly intervals for antibodies. Whenever possible, specimens of cord blood were obtained at the time of delivery. Presence of antibodies was determined simultaneously by the saline agglutination, 20 per cent bovine albumin, and antiglobulin[2] techniques. The cord specimens were grouped and typed, the cells studied with the antiglobulin technique for antibody coating, and the respective sera searched for antibodies with the methods mentioned. The children born of immunized Rh-negative mothers were checked with the routine hematologic methods as well as the antiglobulin technique.

RESULTS

As shown in Table I, over 86 per cent of patients were Negroes. The incidence of d (Rh_0 negative) in Negroes and Caucasians was 7.3 and 17.4 per cent respectively. Among the 197 Rh-negative Negroes, eight were found to be sensitized to Rh, and among the seventy-three Caucasians three were shown to have Rh antibodies. If one expresses these figures in per cent of d (Rh negative) (see Table II), one finds that 4.06 per cent of the Negroes and 4.1 per cent of the Caucasians were sensitized. However, in relating the number of sensitized patients to the total number in each racial group, it may be

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TABLE I

	NEGROES	PER CENT	CAUCASIANS	PER CENT
D (Rh_0 pos.)	2499	92.7	346	82.6
d (Rh_0 neg.)	197	7.3	73	17.4
	2696		419	

shown that 0.3 per cent of the Negroes and 0.7 per cent of the Caucasians had developed Rh antibodies. The ratio of Negro to white Rh negatives in this series is 1:2.4, and the corresponding ratio of sensitized patients, 1:2.7.

TABLE II

d (Rh_0 NEGATIVE)	NEGROES	CAUCASIANS
Total number of Rh-negative patients	197	73
Patients sensitized	8	3
Per cent sensitized	4.06	4.1
Per cent sensitized of entire racial group	0.3	0.7

As shown in Table III, all of the Negro patients were multiparas. One subject (Patient 2) had a definite history and another (Patient 4) a questionable history of erythroblastosis in a previous pregnancy. In four of the eight cases a history of transfusion was elicited. Only Patient 6 received transfusions preceding the first pregnancy. All but one (Patient 8) showed a consistent absence of Rh agglutinins. In five patients the outcome of the pregnancy is known, and at the time of birth all were described as clinically

TABLE III

PATIENT	RACE	PARITY		PREVIOUS INSTANCE OF ERYTHROBLASTOSIS FETALIS	TRANSFUSION HISTORY	MATERNAL RED CELLS		ANTI-Rh			TRIMESTER	CLINICAL APPEARANCE AT BIRTH
		PARA	GRAVIDA			GROUP	Rh TYPE	SALINE	ALBUMIN	ANTIGLOBULIN		
1	Negro	II	III	—	1	O	rh″(dcE)	0	64	128	3rd	Normal
2	Negro	XII	XIV	11th, 12th, and 13th children	–	O	rh(dce)	0	8	128	3rd	Normal
3	Negro	I	III	—	–	O	rh(dce)	0	16	32	3rd	Normal
4	Negro	III	IV	3rd child jaundiced	–	O	rh(dce)	0	16	16	3rd	Normal
5	Negro	III	VI	—	4	O	rh(dce)	0	4	4	3rd	Normal
6	Negro	VIII	VIII	—	6*	B	rh(dce)	0	16	16	3rd	
7	Negro	I	III	—	1	O	rh(dce)	0	16	16	3rd	
8	Negro	I	II	—	–	B	rh(dce)	4	16	16	3rd	
9	Caucasian	V	VIII	—	–	O	rh(dce)	Trace	8	8	3rd	Mild jaundice
10	Caucasian	II	III	2nd child	–	A	rh(dce)	0	8	32	3rd	Normal
11	Caucasian	III	IV	2nd and 3rd children	–	O	rh(dce)	0	32	32	1st	

*Previous to first pregnancy.

†These cells were saturated with incomplete maternal antibody so that they failed to be agglutinated in vitro by anti-Rh_0 agglutinating sera.

normal. There was not a single instance of incompatibility of the major blood groups. However, on laboratory examination, the four cord and/or peripheral blood specimens belonging to Rh-positive children showed antibody coating to a varying extent (antiglobulin technique).

CASE REPORTS

CASE 1.—The clinical appearance of this infant at birth was that of an entirely normal full-term baby. Its red blood cells, however, were "blocked" so that they gave the reaction of Rh_0 negative cells with agglutinating sera. The antiglobulin reagent gave a 4+ reaction on cord and peripheral cells and incomplete antibodies could be demonstrated in the cord serum. Hemoglobin and red blood count shortly after birth were 13.6 grams and 4.3 million respectively. This child was discharged on the third postnatal day because of "apparent" lack of involvement. The subsequent course is summarized in Table IV.

As can be seen from Table IV, this infant's hemoglobin dropped markedly after its discharge, and the infant was brought back to the hospital only because the mother had been warned of the possible complications. There was no evidence of jaundice at any time. Despite four transfusions, the hemoglobin value persistently dropped as long as antibodies could be demonstrated to be attached to the red cells (i.e. approximately seven and one-half weeks postpartum).

CASE 2.—This infant was apparently normal at the time of delivery but on the eighth day was noticed to be jaundiced, pale, and in acute respiratory distress. On admission, hemoglobin was 2 grams and red blood count 610,000 per cubic millimeter. Erythrocytes gave a 4+ antiglobulin reaction, but were not completely "blocked." Incomplete anti-Rh antibodies could be demonstrated in the serum. The subsequent course is summarized in Table V.

TABLE III

BABY'S BLOOD		CORD BLOOD		PERIPHERAL BLOOD			
GROUP	Rh TYPE	ANTI-GLOBULIN TEST ON CELLS	ANTI-BODIES IN SERUM	ANTI-GLOB-ULIN	HGB.	R.B.C.	COURSE
O	"blocked"†	4+	Present	4+	13.6	4.3	Transfused 4 times; now living and well
O	Rh_0(Dce)			4+	2.0 (8th day)	0.6	Transfused 4 times; now living and well
O	"blocked"†	3+	Present	3+	12.0	3.8	Replacement transfusion; now living and well
O	rh'(dCe)			Neg.	12.6		Living and well
O	Rh_0(Dce)	1+	None	1+	15.6		50 days post partum. Hgb. 10 Gm.; antiglobulin negative; now living and well
		Undelivered					
		Outcome unknown					Living and well
O	Rh_1(DCe)	1+	None	1+	Normal limits		Transfused once; now living and well
AB	Rh_1(DCe)	3+	None	2+	17.0		14 days post partum. Hgb. 9.8; R.B.C. 3.1; antiglobulin 1+; transfused once; now living and well
		Undelivered					

TABLE IV. CASE 1

POST-NATAL DAY	ANTI-GLOBULIN TEST	HGB. (GM.)	R.B.C. (MILLIONS)	W.B.C.	NORMO-BLAST	RETICU-LOCYTES	COMMENTS
1	4+	13.6	4.3	21,600	9%	8.6%	
2	4+	13.6					
3	4+	13.7					Discharged
16	4+	8.8	2.8				Readmitted; 85 c.c. transfusion
17	4+	10.7					85 c.c. transfusion
20	4+	10.2					85 c.c. transfusion
21	4+	12.0					
22	4+	14.8					85 c.c. transfusion
23	4+	14.0					
24	4+	13.6					
25	4+	13.6					
27	4+	17.0	5.4	5,300	0		
51	1+	9.9					
71	Neg.	11.0					

As in the first infant, the intensity of the antibody coating of the red cells seemed to parallel the clinical course. It is of interest that the mother of this child lost her four previous children from erythroblastosis. Her ninth and tenth children were severely jaundiced and lived for six and twelve days respectively. Her eleventh and twelfth pregnancies terminated in nine months with stillbirths.

TABLE V. CASE 2

POSTNATAL DAY	ANTIGLOBULIN TEST	HGB.	R.B.C.	COMMENTS
8	4+	2.0	610,000	150 c.c. transfusion (whole blood)
9	1+	2.0		35 c.c. washed cells
10	1+	14.0		
11				70 c.c. washed cells
16	3+	18.8		Patient dehydrated
19				120 c.c. whole blood
21	Neg.			
29	Neg.			Peripheral blood appeared entirely Rh-negative
31	1+	13.5		Patient clinically well
38	Neg.	13.0		Discharged; living and well since

CASE 3.—This infant also was described as clinically normal at birth. Its hemoglobin was 12.0 grams, with a red blood count of 3.8 million per cubic millimeter. Antiglobulin reaction was 4+. Red cells from the cord as well as from the baby's peripheral circulation were completely "blocked" and the cord serum contained incomplete antibodies. Because of our experience with previous similar cases, a replacement transfusion of 500 c.c. was given on the first postnatal day. Antiglobulin reaction after the transfusion was 1+. On the third day hemoglobin had risen to 16.4 grams with a weakly positive antiglobulin test. Thirty days after the replacement transfusion the baby was discharged from the hospital with a hemoglobin of 11.2 grams, red blood count of 4.0 million per cubic millimeter, and a negative antiglobulin reaction. The baby has remained well since.

CASE 4.—This child was clinically Rh_0 negative (dCe; rh') and consequently not affected by the maternal antibodies which were anti-D (Rh_0) in specificity. Antiglobulin reaction on cord cells was negative and the infant remained well despite a somewhat low hemoglobin (12.6 grams) at birth. It is of interest that on a six-week post-partum sample of the mother's blood, anti-rh' (anti-C) agglutinins could be demonstrated.

CASE 5.—This baby was born of a mother possessing a very low incomplete Rh antibody titer. At the time of birth the baby appeared normal and has remained so. Its hemoglobin was 15.6 grams and the antiglobulin reaction on cord cells was only 1+. There were no free antibodies demonstrable in the cord serum. On the fiftieth post-partum day the hemoglobin had dropped to 10.0 grams, and no antibodies could be detected on the surface of the red cells.

CASE 6.—As yet undelivered.

CASE 7.—This was a normal full-term child which has remained well according to the mother. This child was not available to us for testing.

CASE 8.—The outcome of this case is unknown to us, but because of low parity and the low titer of incomplete antibodies in the third trimester, experience indicates that this child probably was not affected.

CASES 9, 10, and 11.—These are the cases observed in Caucasians and are listed only because they occurred within our present series.

DISCUSSION

Because of our findings that Negroes can be apparently immunized against Rh with the same frequency as Caucasians, we proceeded to check our hospital statistics for clinical erythroblastosis in the two races over a period of eight years. These findings are summarized in Table VI.

TABLE VI

YEAR	NUMBER OF HOSPITAL CASES OF ERYTHROBLASTOSIS FETALIS		TOTAL HOSPITAL BIRTHS	
	CAUCASIANS	NEGROES	CAUCASIANS	NEGROES
1941	0	0	697	639
1942	0	0	828	823
1943	0	0	980	850
1944	5	0	1,064	825
1945	5	1	1,267	952
1946	2	0	1,490	1,222
1947	7	1	1,649	1,522
1948	10	5	1,460	1,870
Total	29	7	9,500	8,931

From Table VI it can be seen that over the eight-year period erythroblastosis was diagnosed in Caucasians at a rate of 1 out of approximately 300 births. For the Negroid races this figure, however, was only 1 in 1,300 cases. The ratio of frequency between the two groups is 1:4. It can also be seen that as the years passed the frequency of the diagnosis rose with increasing knowledge of the Rh factor. The majority of cases have been diagnosed during the past two years, over which period our study has extended.

The incidence of erythroblastosis reported by various authors ranges from 1:150 to 1:568 (see Table VII), with an average incidence of about 1:300+. However, it is to be noted that in most statistics cited the Negro cases have been included in the total group.

TABLE VII. INCIDENCE OF ERYTHROBLASTOSIS

AUTHORS	YEAR	INCIDENCE
Diamond[3]	1945	1:150
Ponder, Ponder, and Levine[4]	1948	1:169
Schwartz and Levine[5]	1943	1:200
Potter[6]	1947	1:252
Javert[7]	1942	1:438
Wolfe[8]	1940	1:568

In order to ascertain whether the difficulty in finding cases of erythroblastosis in Negroes was shared by other institutions, a questionnaire* was sent to seven departments of pediatrics in regions with large Negro populations. Four of these institutions reported cases and gave the frequency of Negro births. These are summarized in Table VIII.

TABLE VIII

	CASES OF ERYTHROBLASTOSIS FETALIS, HYDROPS, OR ICTERUS GRAVIS IN NEGROES	DUE TO Rh?	NUMBER OF NEGRO BIRTHS
1. Duval County Hospital, Jacksonville, Fla. (H. A. Carithers)	None	—	488 per yr.
2. Tulane University, New Orleans, La. (R. V. Platou)	5	Yes, 2 due to AB incompatability	6,000 per yr.
3. Duke University, Durham, N. C. (G. Taylor)	1	Yes	535 per yr.
4. Louisiana State University, New Orleans, La. (M. E. Wegman)	3	Yes, also Hr	6,500 per 2 yr.

*Through courtesy of Dr. Lee E. Sutton, Jr., Professor of Pediatrics, Medical College of Virginia.

In this survey seven cases are reported out of 13,523 births if one takes into account only those hospitals which stated their incidence of Negro births and only those cases which were proved to be due to Rh incompatability. This frequency of about 1 in 1,900 births approximates the incidence in our institution (1:1,300).

It remains to be explained why only one case of erythroblastosis is diagnosed in 1,300 births if the rate of immunization to the factor Rh among Negroes is 1 in approximately 330 (0.3 per cent).

As stated by Levine,[9, 10, 11] the true incidence of erythroblastosis fetalis in any given population can be expected to be proportional to the frequency of negative reactions with anti-D (Rh_0) serum. That this apparently holds for immunizations against the Rh factor can be seen from our figures (see Tables I and II). The reasons for the failure to find the expected number of cases of erythroblastosis in Negro infants are probably numerous.

First, not all immunized mothers will have sufficiently potent antibodies to produce clinical symptoms in their offspring.

Second, a certain proportion of these mothers will have Rh-negative, unaffected children.

Third, the pigmented skin makes the diagnosis of icterus and anemia very difficult.

Fourth, in our institution Negro multiparas usually stay in the hospital for only twenty-four to forty-eight hours after delivery, which will remove their offspring from clinical observation during the time symptoms may develop (Cases 1 and 2).

Fifth, many stillborn infants are not autopsied, and erythroblastosis is probably undiagnosed.

Sixth, because marked jaundice was unusual in our group and severe anemia rather common, many cases may have been diagnosed in the past as anemia of the newborn.

Whether or not the disease is actually less severe in Negroes cannot be ascertained from this short series.

SUMMARY

On the basis of a study of 3,115 consecutive obstetric clinic patients of which 86 per cent were Negroes, presumptive evidence is submitted that Rh-negative Negroes are sensitized at the same rate as Caucasians. The total number of sensitized patients is shown to be directly proportional to the frequency of the Rh_0 negative reaction in a given population. The incidence of affected Negro children in our series was about 1:900 pregnancies. Reasons are suggested for the failure to diagnose erythroblastosis in Negroes at the expected rate.

We wish to thank Dr. H. H. Ware, Jr., Professor of Obstetrics, and Dr. Lee E. Sutton, Jr., Professor of Pediatrics, for their kind permission to use the cases cited.

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NEBULIZED PYRIBENZAMINE IN NASAL AND BRONCHIAL ALLERGY

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THE usefulness of the antihistamine drugs in alleviating the symptoms of nasal allergy is now well established. It is the general experience, however, that many patients fail to be relieved and certain symptoms, such as blocking of the nasal passages, are particularly resistant to these drugs. Among possible explanations for the failure to achieve the desired effect by oral therapy is that an insufficient concentration of the compound reaches the nasal tissues. The toxicity of the present series of antihistamine substances limits the amount which may be given orally to produce a higher nasal concentration. Since in earlier investigations we demonstrated[1, 2] that small quantities of the antihistamine compounds when applied to the skin would inhibit the histamine and allergic reaction, it was considered likely that the topical use of these drugs would be effective in skin[3] and respiratory allergy.

In 1945 we began to experiment with nasal drops of antihistamine drugs. We found that a concentration of Pyribenzamine as low as 0.5 per cent was irritating to the nose and particularly the pharynx of many patients. Recent reports[4, 5] claimed favorable results with such drops. The ordinary nasal spray was also objectionable for similar reasons, although both techniques were helpful in some instances. We finally adopted the technique of administering a nebulized solution from a nebulizer (DeVilbiss No. 40) with an attached nasal piece. This provides an aerosolized mist which is nonirritating and reaches the nasal and pharyngeal tissues most readily.

A 2 per cent buffered solution of Pyribenzamine was found the most generally useful concentration. These topical treatments were not employed routinely for the relief of seasonal hay fever or allergic rhinitis, but were confined to those patients who either were not getting sufficient results from oral antihistamine therapy or who could not tolerate the latter. A number of these patients owed their resistant nasal obstruction to the repeated use of topical vasoconstrictors. Although at times the effect of this nebulization was immediately noticeable, more commonly it was not apparent until later, and particularly after several treatments had been given. In other words, the action of this therapy in these severe cases of nasal congestion is more of a preventive nature and requires repeated administration.

A nebulized mist of 2 per cent Pyribenzamine, released by about half a dozen squeezings of the bulb of a DeVilbiss No. 40 nebulizer, and repeated every two or three hours, produced benefit in twenty-seven out of thirty-four patients with the type of nasal congestion described. This relief had not been obtained in the same patients with the oral use of Pyribenzamine or other

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antihistamine compounds. Of two patients who failed to respond to a 2 per cent solution, one responded to a 5 per cent concentration. It should be emphasized that the effect of vasoconstricting drops is more marked and more dramatic than this therapy in relieving nasal obstruction. However, the marked congestion which frequently follows the shrinking effect of the repeated use of potent vasoconstrictors is a penalty which frequently is responsible for the continuation of the nasal blocking. The topical antihistamine therapy does not produce this deleterious effect and for that reason is preferable in these difficult cases.

In bronchial allergy the antihistamine drugs orally are much less effective than in nasal allergy. Acute or severe asthma is almost never relieved by these drugs, while moderate wheezing may be helped. In spasmodic cough due to bronchial allergy, without dyspnea, these compounds orally are effective in about one-third of the patients. From our experience with topical treatment in these cases we have the feeling that here, too, the major difficulty probably is the presence of a greater amount of a chemical mediator than can be combated by oral administration of antihistamine drugs. We have employed aerosols of various antihistamine drugs, using varying strengths of solutions and various methods of application. Here we wish to record briefly only the results with Pyribenzamine solution as nebulized with the ordinary hand nebulizer or oxygen pressure.

Out of fifty-seven patients with asthma, most of whom did not respond to Pyribenzamine by mouth, ten obtained effective relief and thirteen had slight relief with a 2 per cent Pyribenzamine aerosol. It should be emphasized that the vast majority of this group were ambulatory patients, appearing at the office, and presenting a moderate rather than severe asthmatic state. A 1 per cent aerosol was effective in three out of eight patients, while a 5 per cent aerosol was effective in five out of nine patients. The spasmodic cough without dyspnea appeared to respond better than the true asthma, twelve out of sixteen patients reacting favorably. In most instances this type of therapy was less effective than aerosol therapy with epinephrine or isopropyl epinephrine. In several cases of cough, the antihistamine aerosol gave better results than these bronchodilators. The main advantage of the use of the antihistamine drugs by aerosol, however, rests on the much lower toxicity of these solutions and their consequent safety in frequent use, as compared with the other aerosols and the antihistamine drugs orally.

SUMMARY

The results of the application of aerosolized 2 per cent Pyribenzamine solutions to the nose and bronchi indicate that this is a useful adjunct method in the treatment of selected cases of nasal and bronchial allergy.

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THE ADAPTABILITY OF MICE TO THE LABORATORY DIAGNOSIS OF TUBERCULOSIS

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THE mouse is ordinarily considered to be resistant to induced tuberculous infection. In reviewing the literature one is at once impressed by the lack of detailed information regarding experimental tuberculosis in mice. Moreover, reports by the various investigators reveal a wide divergence of opinion relative to the existence of any reliable and constant characterization of the infection in these animals.[1-10]

Recent studies have stimulated interest in the possibility of using mice for the evaluation of antituberculosis agents[11-15, 22] and for the identification of virulent acid-fast bacilli. The latter investigations, conducted by Dubos and associates,[16, 19] have shown that pigmented (line 1 dba and C 57) strains of mice appeared to be more susceptible to tuberculous infection than other strains examined and, moreover, young cultures grown diffusely in certain liquid media were found to be extremely infective for the mice. It was observed, too, that deficient (corn meal) diets,[17] and also concurrent infection with pneumotropic viruses,[18] appeared to accelerate the course of pulmonary tuberculosis in mice.

In light of this recent work, the Tuberculosis Evaluation Laboratory has undertaken a series of experiments designed to evaluate the reliability of mouse inoculation tests, with special emphasis upon their usefulness in the routine laboratory diagnosis of tuberculosis.

MATERIALS AND METHODS

Mice.—Mice of the C57 black and dba strains[16] were obtained from Rockland Farms and at the time of inoculation varied in age from 2 to 11 weeks. Immediately after inoculation the mice were placed in metal cages with wood shavings and wire mesh tops, in most instances four or six animals per cage. In the preliminary experiment to be reported, and for general maintenance, the mice were fed regularly a diet of mouse pellets‡ and water, but in all subsequent experimental work corn meal[17] was substituted at the time of inoculation.

Cultures.—For preliminary work in the development of techniques, standard strains§ of human (H37 Rv), bovine (Ravenel), and avian (Kirchberg) tubercle bacilli were used. These stock cultures were maintained in a Tween-albumin medium‖ patterned after the liquid medium of Dubos[20] in which, after six to seven days, growth appeared as a diffuse, fairly homogeneous suspension containing 0.2 to 0.3 mg. of bacilli (dry weight) per milliliter of medium. Saprophytic acid-fast bacilli were used in certain of the experiments as

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‖Generously supplied by Difco Laboratories, Detroit, Mich.

controls, and these consisted of organisms recently isolated in this laboratory from sputum specimens. They were avirulent for the guinea pig. The timothy bacillus *(Mycobacterium phlei)* was not used in mouse infection tests involving the liquid medium, since observations here had shown it to grow in a nonacid-fast form. A similar observation has previously been reported by Dubos and co-workers[19] in regard to another avirulent strain (H37 Ra). One BCG (Holm) strain was also used, as well as several organisms recently isolated on modified[21] Löwenstein's medium from sputa and gastric washings.

Inoculation and Autopsy.—Cultures grown diffusely in the liquid medium were inoculated into mice in 0.05 ml. volumes, either directly or diluted to the desired dosage with sterile saline. The modes of infection utilized were (a) intravenously in the tail vein or (b) intracerebrally in the left hemisphere while the animal was under ether anesthesia. Growth from the surface of Löwenstein slants was prepared for mouse inoculation by grinding a weighed sample in a sterile mortar with sterile saline in such a manner that the desired dosage was suspended in 0.05 milliliter. Autopsies were performed on all animals dying during the experimental period (these deaths were attributable, in some part, to intercurrent infections) as well as on all survivors, the latter being sacrificed at the conclusion of the experiments, usually one to four weeks after infection. Evaluations of virulence were based upon the ability of the organisms to proliferate and invade spleen, lung, liver and, in the case of intracerebral inoculations, brain tissue. For purposes of these evaluations, impression smears were prepared from the organs listed and stained by the Ziehl-Neelsen technique, special care being taken, in the case of brain tissue, to prepare smears from the cerebral hemisphere opposite the site of inoculation so that some measure of the degree of spread of the infection within the brain would be available. Arbitrarily, three lengths of each smear were examined microscopically and a record was kept of the total number of acid-fast bacilli seen. To express more graphically the extent of the bacterial invasion, the following microscopy code was adopted, based upon the total bacterial count:

1 – 20	=	+
21 – 50	=	++
51 – 100	=	+++
101 or more	=	++++

Gross lung lesions were designated as being either present (+) or absent (–).

In all instances in which the virulence of the organisms had not been determined previously, guinea pig controls were inoculated simultaneously in the right thigh by the subcutaneous route. The extent of infection was recorded in the following manner, according to the distribution of gross lesions noted at the time of autopsy and confirmed by the demonstration of acid-fast bacilli microscopically:

Inguinal lymph node	=	Tub. 1
All lymph nodes	=	Tub. 2
Lymph nodes + spleen	=	Tub. 3
Lymph nodes + spleen + liver	=	Tub. 4
Lymph nodes + spleen + liver + lungs	=	Tub. 5

Experiment 1.—The first experiment was set up with the purpose of deriving a standard technique of mouse inoculation and autopsy for use in subsequent experimental work.

Twenty-four dba mice, 10 to 11 weeks of age, were used, half of which were inoculated intravenously and half intracerebrally. Within each series of twelve mice, six received 0.01 mg. and six, 0.0001 mg. of an H37 Rv culture grown in the Tween-albumin medium and injected in a total volume of 0.05 milliliter. In this preliminary experiment it was reasoned that it might be of some value for future comparisons with mice transferred to the deficient diet, to maintain the present animals on regular mouse pellets. At intervals of one, two, and four weeks, two mice in each series of six were killed and autopsied. The lungs of all animals were examined carefully for gross lesions, and at the same time im-

pression smears of lung tissue were stained and examined for the presence of acid-fast bacilli. Supplementary smears were prepared of brain tissue in the case of those mice inoculated intracerebrally, and of spleen and liver in those inoculated intravenously.

Results of Experiment 1: Results presented in Table I reveal that bacilli inoculated intravenously in a dose of 0.01 mg. can be recovered in lung, liver, and spleen smears as early as one week after inoculation. Lung smears, however, appeared to be inferior from the standpoint of the number of organisms that could be demonstrated early (one to two weeks) in the disease. With the smaller dose (0.0001 mg.) there was noted a marked superiority for spleen smears, the only organ in which bacilli could be detected in one week. This superiority was present fairly consistently through the entire experiment.

TABLE I. RESULTS OF THE INFECTION OF DBA MICE WITH VIRULENT (H37 RV) TUBERCLE BACILLI BY THE INTRAVENOUS ROUTE

DOSE (MG.)	NUMBER OF MICE	AUTOPSY FINDINGS* 1 WK. LUNG	1 WK. SPLEEN	1 WK. LIVER	1 WK. GROSS LUNG LESIONS	2 WK. LUNG	2 WK. SPLEEN	2 WK. LIVER	2 WK. GROSS LUNG LESIONS	4 WK. LUNG	4 WK. SPLEEN	4 WK. LIVER	4 WK. GROSS LUNG LESIONS
0.01	6	+	++++	++++	−	+	++++	++++	−	++++	++++	++++	−
		+	++++	++++	−	+	++++	++++	−	++++	++++	++++	−
0.0001	6	−	+	−	−	+	+	+	−	+	++++	+	−
		−	+	−	−	−	++++	+	−	+	++++	+	−

*For explanation of symbols see text.

The results of the intracerebral inoculations are summarized in Table II. As in the case of infection by the intravenous route, the initial time of appearance of pulmonary involvement was in fairly close relationship to the size of the inoculum. However, invasion of the cerebral hemisphere opposite the site of inoculation was detectable in the first week regardless of the dosage.

It will be noted that in none of the mice could macroscopic lung lesions be demonstrated, nor were there any deaths as a result of the tuberculous infection.

On the basis of results obtained in this preliminary study the intracerebral route of inoculation was adopted in all subsequent experiments since, by this method, pulmonary involvement was shown to take, essentially, the course observed after infection by the intravenous route. Moreover, the former technique was preferred because of the relative ease and rapidity with which it can be performed.

TABLE II. RESULTS OF THE INFECTION OF DBA MICE WITH VIRULENT (H37 RV) TUBERCLE BACILLI BY THE INTRACEREBRAL ROUTE

DOSE (MG.)	NUMBER OF MICE	AUTOPSY FINDINGS* 1 WK. BRAIN	1 WK. LUNG	1 WK. GROSS LUNG LESIONS	2 WK. BRAIN	2 WK. LUNG	2 WK. GROSS LUNG LESIONS	4 WK. BRAIN	4 WK. LUNG	4 WK. GROSS LUNG LESIONS
0.01	6	++++	+	−	++++	+	−	+++	++++	−
		++++	+	−	++++	++	−	+	+++	−
0.0001	6	++	−	−	−	+	−	++	−	−
		+++	−	−	+	+	−	†		

*For explanation of symbols see text.

†One mouse died in twenty-four hours and was eaten by survivors so that no tissue examination was possible.

Experiment 2.—In this experiment an attempt was made to determine (a) whether tubercle bacilli of all three types are virulent for the mouse, and (b) whether there is any perceptible difference between the three types in respect to their mouse pathogenicity. Young (7-day-old) cultures growing diffusely in the Tween-albumin medium were used throughout. Strains of tubercle bacilli tested consisted of an avian (Kirchberg), a human (H37 Rv), a bovine (Ravenel), and an acid-fast saprophyte recently isolated from sputum and avirulent for the guinea pig. With each strain, six mice of the C57 strain, 4 weeks of age and maintained on a diet of corn meal and water from the time of infection, were inoculated intracerebrally with 0.05 ml. of undiluted culture (approximately 0.01 mg. dry weight). At one-, two-, and four-week intervals some of the animals were sacrificed and autopsied, at which time the lungs were examined for evidence of gross lesions. Impression smears were prepared of brain, spleen, and lung tissue. Five of the twenty-four mice died during the experimental period.

Results of Experiment 2: As indicated in Table III, all three virulent types produced much the same picture on autopsy. As early as seven days there were signs of marked multiplication of tubercle bacilli in the brain tissue and an invasion of the spleen. In two weeks bacilli were present in large numbers in the lung smears of all animals examined, but only in those inoculated with the bovine strain were there grossly visible lung lesions. These lesions appeared first in those mice killed at the end of two weeks. In none of the six mice inocu-

TABLE III. RESPONSE OF C57 MICE TO INOCULATION WITH VIRULENT (BOVINE, HUMAN, AVIAN) AND SAPROPHYTIC ACID-FAST BACILLI BY THE INTRACEREBRAL ROUTE

CULTURE	DOSE (MG.)	NUMBER OF MICE	AUTOPSY FINDINGS*				
			DAYS UNTIL AUTOPSY†	BRAIN	SPLEEN	LUNGS	GROSS LUNG LESIONS
Avian (Kirchberg)	0.01	6	K-7	++++	++++	–	–
			K-7	++++	++++	–	–
			D-7	++++	++++	+	
			K-14	++++	++++	++	–
			K-29	++++	++++	++++	–
			K-29	++++	++++	++++	–
Human (H37 Rv)	0.01	6	K-7	++++	++++	–	–
			K-7	++++	++++	–	–
			D-14	+++	++++	++++	–
			K-14	++++	++++	++	–
			D-18	+	++++	++++	–
			K-28	++++	++++	++++	–
Bovine (Ravenel)	0.01	6	K-7	++++	++++	+	–
			K-7	++++	++++		–
			K-14	++++	++++	++++	+
			K-14	++++	++++	++++	+
			D-19	++++	++++	++++	+
			D-24	++++	++++	++++	+
Saprophyte	0.01	6	K-7	+	–	–	–
			K-7	++	–	–	–
			K-14	–	–	–	–
			K-14	+	–	–	–
			K-28	+	–	–	–
			K-28	–	–	–	–

*For explanation of symbols see text.
†D=death, K=killed; the numeral indicates the number of days after inoculation.

lated with the saprophyte was it possible to recover acid-fast bacilli by smear from the spleen or lung, and only an occasional organism was observed in the brain tissue.

The results presented in Table III reveal, then, that the C57 mice were susceptible to the three types of virulent tubercle bacilli inoculated intracerebrally. Any conclusion, however, regarding the adaptability of the mice to the type determination of tubercle bacilli could hardly be justified on the basis of the present findings. There were observable differences from the standpoint of certain criteria (deaths, gross lung lesions, relative numbers of bacilli and their time of appearance), but these were not thought to be significant enough for type differentiation.

Experiment 3.—In the foregoing experiments the results appeared to be encouraging enough to warrant further investigations, particularly in respect to a search for a practical application of mouse infection tests in the routine laboratory diagnosis of tuberculosis.

Most laboratories today utilize the classical solid egg media, or modifications of them, in the primary isolation of tubercle bacilli from pathologic material. In order to simulate routine conditions, all cultures tested in the experiment to be described were grown on a modified Löwenstein medium as recommended by Holm.[21] In all, twenty-five strains of acid-fast bacilli were studied and these included twenty primary isolations from sputum specimens and one from a gastric washing. For purposes of control, cultures of standard human (H37 Rv), bovine (Ravenel), saprophytic (timothy), and attenuated mammalian (BCG, Holm) organisms were also included. Mice of the C57 and dba strains (7 to 10 weeks of age) were inoculated intracerebrally with 0.01 mg. of bacilli suspended in 0.05 ml. of sterile saline. Guinea pig controls received the same dosage subcutaneously in the right thigh. The mice were maintained on a diet of corn meal and water from the time of infection, three to four animals of the same age and strain being used for each of the twenty-five cultures.

As shown in Table IV, bacilli cultivated on the solid medium appeared only in the brain tissue of certain of the mice sacrificed at the end of one week. This suggested that these animals might have been killed prematurely and for that reason the period of infection, for survivors, was extended to twenty-one days in the remainder of the experiment. At the time of autopsy, impression smears were made of brain, lung, and spleen tissue and a record was kept of the presence of macroscopic pulmonary lesions. The guinea pig controls were killed and autopsied at the end of six weeks.

Results of Experiment 3: Table IV shows that twenty-one of the twenty-five strains studied proved to be virulent for the guinea pig according to the usual criteria. In the mice which had received the corresponding organism in the same dosage, there was, in general, evidence of bacterial proliferation within the brain tissue as well as an invasion of spleen and lung. It will be noted, however, that there occurred some marked inconsistencies, at autopsy, within groups of animals which had received the same culture in supposedly the same dosage. This phenomenon had been observed, but to a lesser extent, in the preceding experiments. Of the factors which might be postulated to have had some bearing on these discrepancies, any one, or a combination, of the following might conceivably have been responsible: (a) inconstant dosage because of the presence of clumps of bacilli in both mechanically triturated suspensions and cultures growing diffusely in liquid media[16]; (b) the impossibility, in intracerebral inoculations, of depositing organisms in the same position within the

TABLE IV. RELATIVE VIRULENCE, FOR MICE AND GUINEA PIGS, OF CULTURES OF ACID-FAST BACILLI

CULTURE*	AGE OF CULTURE (DAYS)	MICE					GUINEA PIGS	
		DAYS UNTIL AUTOPSY†	BRAIN	LUNG	SPLEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY†	AMOUNT OF TUBERCULOSIS‡
H37 Rv	21	K-21	+	−	−	−	D-35	Tub. 3
		K-21	++++	+	+++	−		
		K-21	++++	−	++	−		
		K-21	++++	++++	++++	−		
Bovine (Ravenel)	11	D-16	−	−	++	−	K-42	Tub. 5
		K-21	++	++++	++	+		
		K-21	−	−	+	−		
		K-21	++++	++++	+	−		
Timothy	6	K-21	−	−	−	−	K-42	−
		K-21	−	−	−	−		
		K-21	−	−	−	−		
		K-21	−	−	−	−		
BCG (Holm)	34	D-13	−	−	−	−	K-42	−
		K-21	−	−	−	−		
		K-21	−	−	−	−		
		K-21	+	−	−	−		
S-1045	36	K-7	−	++	++++	−	K-42	Tub. 3
		K-7	−	−	−	−		
		K-21	++++	−	++	−		
		K-21	++++	+	+++	−		
S-1058	35	K-7	+	−	−	−	K-42	Tub. 3
		K-7	++++	−	−	−		
		K-21	++	+	+	−		
		K-21	++++	−	+	−		
S-1074	32	K-7	+	−	−	−	K-42	Tub. 3
		K-7	++++	−	+	−		
		K-21	++	++++	+++	−		
		K-21	++	++	+	−		
S-604	48	K-7	−	−	−	−	K-42	−
		K-7	−	−	−	−		
		K-21	−	−	−	−		
		K-21	−	−	−	−		
G-55	60	K-7	−	−	−	−	K-42	Tub. 3
		D-7	+	−	−	−		
		K-21	++++	+	++++	−		
		K-21	+++	−	+	−		
S-1165	25	K-7	++	−	−	−	K-42	Tub. 3
		D-7	++	−	−	−		
		D-15	++++	−	++++	−		
		K-21	++++	−	+	−		
S-701	29	D-1	+	−	−	−	K-42	−
		D-7	+	−	−	−		
		K-21	++	−	−	−		
		K-21	+++	−	−	−		
S-1113	34	K-21	++++	++++	+	−	D-20	Tub. 1
		K-21	++++	−	+	−		
		K-21	+++	+	+	−		
		K-21	++++	++++	+++	−		
S-1133	30	K-21	++++	+	+	−	K-42	Tub. 3
		K-21	+	−	+	−		
		K-21	+++	−	−	−		
		K-21	+++	−	+	−		

*S=sputum specimen, G=gastric specimen.
†D=death, K=killed; the numeral indicates the number of days after inoculation.
‡For explanation see text.

TABLE IV.—CONT'D

CULTURE*	AGE OF CULTURE (DAYS)	MICE					GUINEA PIGS	
		DAYS UNTIL AUTOPSY†	BRAIN	LUNG	SPLEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY†	AMOUNT OF TUBERCULOSIS‡
S-1122	34	K-21	++++	++++	+++	−	K-42	Tub. 3
		K-21	++++	++++	+++	−		
		K-21	++++	+	++	−		
		K-21	+++	++++	++++	−		
S-1140	36	K-21	++++	++++	++++	+	K-42	Tub. 5
		K-21	++++	++++	++++	+		
		K-21	+	+	++	−		
		K-21	−	−	−	−		
S-1146	34	K-21	++++	+	+	−	D-26	Tub. 1
		K-21	++++	+	+	−		
		K-21	−	−	+	−		
		K-21	++++	++++	+++	−		
S-1026	29	K-21	++++	+	+++	−	K-42	Tub. 3
		K-21	++++	−	+++	−		
		K-21	++++	−	+	−		
		K-21	++++	−	+	−		
S-1226	28	K-21	++	+	++	−	K-42	Tub. 1
		K-21	+++	−	+	−		
		K-21	++++	−	++	−		
S-1225	28	K-21	++++	−	−	−	K-42	Tub. 1
		K-21	++++	+++	+	−		
		K-21	++++	+	+	−		
S-1270	25	K-21	++++	+	+	−	K-42	Tub. 1
		K-21	+	++++	++	+		
		K-21	+	−	−	−		
		K-21	++	+++	+	+		
S-1299	22	K-21	−	+	+	−	K-42	Tub. 3
		K-21	+	++	+	−		
		K-21	++++	−	+	−		
		K-21	+	++++	++	−		
S-1297	22	K-21	+	++++	++	+	K-42	Tub. 3
		K-21	+	++++	+	+		
		K-21	+	++++	++	+		
		K-21	−	++++	+	+		
S-1168	39	K-21	+	++	+	−	K-42	Tub. 3
		K-21	+	−	+++	−		
		K-21	+	++++	+++	+		
S-1253	28	K-21	+++	+	+++	−	K-42	Tub. 3
		K-21	+	++++	+	+		
		K-21	++++	++++	++++	+		
		K-21	+	++++	++	−		
S-1250	28	K-21	++	−	−	−	K-42	Tub. 1
		K-21	++++	−	−	−		
		K-21	++	+	+	−		
		K-21	−	−	−	−		

brain of every mouse and the relationship this might possibly have to the rate of bacterial spread; (c) the usual errors of random sampling encountered in the selection of material for smear and in the choice of fields in microscopic examinations; and finally (d) it can be assumed, with some degree of certainty, that mice, even though they be of the same age and strain, probably vary considerably in their native susceptibility to tuberculous infection.

From an analysis of the data presented in Table IV it can be concluded, nevertheless, that, in the strains of acid-fast bacilli examined and on the basis

of the evaluation procedure established for the determination of pathogenicity in the present study, there was evidence of close correlation between virulence for mice and virulence as measured by classical guinea pig tests.

Experiment 4.—The results presented in the preceding experiments indicated that a combination of rapid, diffuse culture in a liquid medium, plus subsequent mouse inoculation, would reduce considerably the time required for the diagnosis of tuberculosis in the routine laboratory. Experiment 4 was designed to explore this possibility.

A recent report by Foley[23] described the successful use of the Dubos medium for the rapid, primary isolation of tubercle bacilli from various pathologic materials. Special note, however, was made of the fact that the medium supports the growth of a variety of nonacid-fast organisms. Of considerable interest, then, was the work of Goldie[24] in which the addition of penicillin (0.5 to 2 units per milliliter) to the Dubos medium was advocated in light of its suppressive effect on the growth of contaminants.

For this experiment a total of nine sputum specimens was selected at random. A direct smear was prepared of each specimen and a 2 ml. portion was prepared for culture according to the classical NaOH concentration technique.

With a sterile Pasteur pipette, approximately 0.25 ml. of each neutralized (2N HCl) sediment was inoculated into a tube containing 5 ml. of the Tween-albumin medium, to which had been added penicillin in the amount of 2 units per milliliter. To serve as controls, identical inocula of each sediment were spread evenly over the surface of two tubes of the Löwenstein medium.

After incubation periods of eight, ten, fourteen, fifteen, and twenty-one days certain of the Tween-albumin cultures were prepared for animal inoculation by first centrifuging 3 ml. samples at 3,000 r.p.m. for forty-five minutes. The supernatant fluid was discarded and 0.5 ml. of sterile saline was added to the sediment. Dba mice, varying in age from 8 to 11 weeks and maintained on a diet of corn meal and water from the time of inoculation, were inoculated intracerebrally with 0.05 ml. of the saline suspension, four animals being used for each of the nine cultures. The experimental period was twenty-one to twenty-six days, at the end of which time all the mice were sacrificed and autopsied according to the technique described in the preceding experiments. Four of the thirty-six mice died during the observation period. Guinea pig controls received 0.05 ml. of the saline suspensions subcutaneously in the right thigh and were autopsied after an infection period of six weeks.

It is interesting to note that a preliminary examination of the direct smears of the original sputum specimens revealed that five were positive for acid-fast bacilli, whereas four were negative.

Results of Experiment 4: The results are summarized in Table V. It can readily be seen that penicillin in a concentration of 2 units per milliliter in the Tween-albumin medium was toxic to the tubercle bacilli contained in the experimental inocula. These results stand in sharp contrast to those of Goldie previously described, but would appear to coincide well with the investigations of Kirby and Dubos[25] which revealed that small inocula of tubercle bacilli are highly susceptible to concentrations of penicillin as low as 1 unit per milliliter in Tween-albumin medium. Reports of results from other laboratories,[26] where comparative studies have been made, have advised against the routine use of liquid media in the isolation of tubercle bacilli from pathologic material. Such findings bear out the observations of Dubos[27] who, following a recent evaluation of his own medium, concluded that, "It would appear unwise . . . . to formulate in final form the composition of media best adapted for diagnostic work until more is known of the growth requirements of the different strains of tubercle bacilli which occur in pathological material."

TABLE V. RESPONSE OF dba MICE TO THE INTRACEREBRAL INOCULATION OF PRIMARY SPUTUM CULTURES IN TWEEN-ALBUMIN-PENICILLIN (2 UNITS/ML.) MEDIUM; SUMMARY OF RESULTS OBTAINED WITH 9 RANDOM SPUTUM SPECIMENS

MICROSCOPY (DIRECT SMEAR)	AUTOPSY FINDINGS: ANIMALS INOCULATED WITH TWEEN-ALBUMIN-PENICILLIN CULTURES		LÖWENSTEIN CULTURE CONTROLS (WITHOUT PENICILLIN)
	MICE (21-26 DAYS)	GUINEA PIGS (42 DAYS)	
Number positive = 5	–	–	+*
Number negative = 4	–	–	–

*All five positive cultures were shown to be virulent human type tubercle bacilli by subsequent guinea pig inoculation.

Further investigations into the possibility of deriving a more perfect combination of rapid, diffuse culture from pathologic material with mouse confirmation await, then, the formulation of a reliable liquid medium with the ability to initiate growth of minimal inocula of tubercle bacilli quickly and uniformly and, of equal importance, the ability to eliminate all contaminating microorganisms.

Experiment 5.—The final study in the present series was devoted to an evaluation of the adaptability of mice to the isolation of tubercle bacilli directly from pathologic material. A survey of the literature reveals that investigations of this nature have been neglected. Only recently[16] has there been reported the successful establishment of a tuberculous infection in mice by the injection intracerebrally of 0.01 ml. of sputum obtained from tuberculous patients.

In the following experiment, two routine sputum specimens (S-2205 and S-2206) were prepared for mouse inoculation according to the procedure previously outlined (Experiment 4). After neutralization with 2N HCl, approximately 0.25 ml. of the sediment was seeded on each of two Löwenstein slants and the balance was transferred aseptically to an inoculating bottle containing 1 ml. of sterile distilled water. Mice of the dba strain, 2 to 5 weeks of age, were inoculated intracerebrally with 0.05 ml. of this suspension, four animals being used for each specimen. All mice were maintained on the deficient diet during the experimental period. The unused portion of each suspension was inoculated subcutaneously into the right groin of a control guinea pig. Infection periods and autopsy techniques for both mice and guinea pigs were essentially those described in the preceding experiments.

As can be seen from the data presented in Table VI, these two digested (NaOH) and neutralized (HCl) sputum specimens proved to be extremely toxic to the mice. Only three of the eight animals inoculated survived the twenty-one day infection period. It might be worth mentioning that one additional unrecorded sputum sample killed all four mice instantly. In an effort to determine whether this toxicity might be due to some factor present in untreated material, the balance of this study was confined to an observation of the effect on the mice of clear, catheterized urine specimens which ordinarily require no NaOH homogenization. Each specimen was first centrifuged at 3,000 r.p.m. for fifteen minutes, following which the supernatant fluid was removed. Procedures for culture and for mouse and guinea pig inoculations were identical with those outlined for the sputum specimens. In Table VI these specimen numbers are preceded by the letters "BF." This study was terminated when

it was recognized that the untreated urines were similarly toxic to the mice. Of a total of thirty-six animals, twenty (55 per cent) died during the experimental period.

TABLE VI. RESULTS OF THE INOCULATION OF ROUTINE CLINICAL SPECIMENS SIMULTANEOUSLY INTO MICE, GUINEA PIGS, AND CULTURE MEDIA; MOUSE DOSAGE = 0.05 ML. OF DILUTED (DISTILLED WATER) SEDIMENT INTRACEREBRALLY

		AUTOPSY FINDINGS							
		MICE					GUINEA PIGS		
SPECIMEN	TYPE OF SPECIMEN	NUMBER DEAD/TOTAL	BRAIN	LUNG	SPLEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY†	AMOUNT TBC.‡	CULTURES
S-2205	Sputum	3/4	0.5+*	0	0	–	K-42	–	–
S-2206	Sputum	2/4	3.0+	0.5+	2.0+	–	K-42	Tub. 1	+
BF-265	Urine	3/4	0	0	0		K-42	–	
BF-266	Urine	1/4	0	0	0		K-42	–	–
BF-271	Urine	1/4	0	0	0	–	K-42	–	–
BF-274	Urine	3/4	0	0	0		K-42	–	–
BF-276	Urine	4/4	0	0	0	–	K-42	–	
BF-277	Urine	1/4	0	0	0	–	K-42	–	–
BF-278	Urine	3/4	0	0	0		K-42	–	–
BF-284	Urine	2/4	0	0	0	–	K-42	–	–
BF-292	Urine	2/4	0	0	0	–	K-42	–	–

*In this and subsequent tables this numeral indicates the average of the 0 to ++++ ratings of the individual mice. Autopsies were not performed on those mice which died at the time of inoculation.

†K=killed; the numeral indicates the number of days after inoculation.

‡For explanation see text.

In light of the fact, however, that in one instance (S-2206) the results following mouse inoculation were confirmed by guinea pig inoculation and culture and in another (S-2205) the results indicated a possible superiority for mouse inoculation had the infection period been extended, it was thought that it might be of some value to attempt to minimize or perhaps eliminate the toxic manifestations by introducing certain changes in the technique. With this in mind a new series of ten random pathologic specimens was prepared for animal inoculation and culture by the NaOH concentration technique. Procedures for animal inoculation, autopsy, and culture were identical to those described with the exceptions that sterile physiologic saline was substituted for distilled water as a diluent, the volume of the intracerebral inoculum was reduced from 0.05 ml. to 0.03 ml., and the infection period for mice was extended to six weeks.

Results of Experiment 5: The results are summarized in Table VII. It will be noted that two of the specimens (BF-223 and G-180) killed all four mice instantly. As shown in the table, a total, then, of seventeen (42 per cent) mice died during the six-week infection period. This indicated only a slight reduction in the casualty rates in spite of the modified technique and it seems reasonable to assume that this persistently high percentage of premature deaths will markedly limit the usefulness of mice in the isolation of tubercle bacilli directly from pathologic materials by the methods described. Moreover, the failure, in two instances (S-1365 and G-179), of definitive mouse confirmation of the guinea pig and culture results would seem to indicate that the extended infection period was of no particular advantage. It is interesting to note at this point that the pronounced toxicity of clinical specimens for mice recently has been observed in another laboratory.[28]

TABLE VII. RESULTS OF THE INOCULATION OF ROUTINE CLINICAL SPECIMENS SIMULTANEOUSLY INTO MICE, GUINEA PIGS, AND CULTURE MEDIA; MOUSE DOSAGE = 0.03 ML. OF DILUTED (SALINE) SEDIMENT INTRACEREBRALLY

SPECIMEN	TYPE OF SPECIMEN	AUTOPSY FINDINGS							CULTURES
		MICE					GUINEA PIGS		
		NUMBER DEAD/ TOTAL	BRAIN	LUNG	SPLEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY	AMOUNT TBC.	
BF-220	Swab	2/4	0	0	0	–	K-42	–	–
BF-218	Pleural fluid	1/4	0	0	0	–	K-42	–	–
S-1366	Bronchial washing	1/4	0	0	0	–	K-42	–	–
S-1365	Bronchial washing	2/4	0	0	0.2+	–	K-42	Tub. 1	+
G-175	Gastric	1/4	0	0	0	–	K-42	–	–
G-178	Gastric	1/4	0	0	0	–	K-42	–	–
G-179	Gastric	0/4	0	0	0	–	K-42	Tub. 3	+
BF-224	Urine	1/4	0	0	0	–	K-42	–	–
BF-223	Urine	4/4					K-42		–
G-180	Gastric	4/4					K-42	–	

Experiment 6.—A final experiment was devised to test the possibility of rendering pathologic materials less toxic for the mice by (a) increasing the volume of saline diluent and (b) washing the sediment. For this purpose six microscopically positive sputum specimens were digested (NaOH) and concentrated and to each neutralized (2N HCl) sediment were added 4 ml. of sterile physiologic saline. Half (2 ml.) of each sediment was then transferred aseptically to a sterile centrifuge tube and the material centrifuged for fifteen minutes at 3,000 r.p.m. After removal of the supernatent fluid the material was resuspended in 2 ml. of fresh sterile saline and a portion of each (washed and unwashed) sediment was used for the inoculation of mice, guinea pigs, and culture media. The intracerebral mouse inoculum was 0.03 ml. and the infection period was twenty-one days.

The data summarized in Table VIII reveal a sharp reduction in the number of nontuberculous deaths among the mice. Only eleven (22 per cent) animals died during the twenty-one day experimental period. All of these deaths occurred within a period of from one to fourteen days after inoculation. However, an examination of the findings also reveals that in none of the mice was there evidence of generalized tuberculosis (note the absence of both gross and microscopic lung pathology), although guinea pig results had indicated the presence of virulent tubercle bacilli in all but one specimen (S-976). Moreover, it would appear that, in general, the degree of infection among mice inoculated with washed sediments was less than that in the group receiving unwashed material. It seems probable, then, that some diminution in the number of bacilli occurred as a result of the dilution and washing techniques employed. This disadvantage, although probably slight in the case of specimens containing large numbers of organisms, would gain increasing significance in the case of those materials containing only a few bacilli. Any significant number of failures would constitute, then, what is probably the most valid objection to this method as a practical means of using mice for the isolation of tubercle bacilli directly from pathologic materials.

A recent study of particular significance which should be considered here is that of Milzer and Levine[29] who, using pigmented strains of mice, reported several successful isolations of tubercle bacilli from clinical specimens (sputa and feces) ten to fifteen days after the intraperitoneal inoculation of suspensions containing gastric mucin, rather than saline, as a diluent. No mention was made of any toxic effect on the mice.

TABLE VIII. RESULTS OF THE INOCULATION OF ROUTINE SPUTUM SPECIMENS SIMULTANEOUSLY INTO MICE, GUINEA PIGS, AND CULTURE MEDIA; COMPARISON OF UNWASHED (A) AND WASHED (B) SEDIMENTS

SPECIMEN	SEDIMENT	AUTOPSY FINDINGS: MICE: NUMBER DEAD/TOTAL	MICE: BRAIN	MICE: LUNG	MICE: SPLEEN	MICE: GROSS LUNG LESIONS	GUINEA PIGS: DAYS UNTIL AUTOPSY	GUINEA PIGS: AMOUNT TBC.	CULTURE
S-676	a	0/4	4.0+	0	2.5+	–	K-42	Tub. 5	+
	b	2/4	1.5+	0	1.0+	–	K-42	Tub. 4	+
S-680	a	1/4	3.3+	0	2.7+	–	K-42	Tub. 5	+
	b	1/4	3.0+	0	3.0+	–	K-42	Tub. 5	+
S-681	a	1/4	1.5+	0	1.0+	–	K-42	Tub. 5	+
	b	0/4	1.7+	0	0.7+	–	K-42	Tub. 5	+
S-912	a	0/4	1.0+	0	0	–	K-42	Tub. 3	+
	b	1/4	0	0	0	–	K-42	Tub. 3	+
S-961	a	1/4	1.2+	0	0.5+	–	K-42	Tub. 5	+
	b	2/4	0.2+	0	0	–	K-42	Tub. 3	+
S-976	a	0/4	0.5+	0	0	–	K-42	–	+
	b	2/4	0	0	0	–	K-42	–	+

DISCUSSION

From time to time in the past, and with renewed vigor in recent years, the possibility has been suggested that mice might be found useful in certain types of investigations in the field of tuberculosis. Only one of these types of investigation has been considered here, namely, the detection of virulent tubercle bacilli in pathologic materials. It should be emphasized that sporadic attempts to develop techniques whereby the mouse might conceivably supplant the guinea pig as a criterion of virulence have been made ever since the discovery of the tubercle bacillus by Koch in 1882. It is probably because marked discrepancies were revealed by the various workers that nothing of a practical nature has materialized. In addition, certain other objections have been raised against the mouse as a test animal in the diagnosis of tuberculosis. It has been suggested,[30] for example, that the failure of the mouse to develop tuberculin sensitivity renders the detection of spontaneous tuberculous infections in mice impossible. This disadvantage gains increasing significance in light of the work of Glover,[10] who determined that the inhalation, by mice, of as few as fifty to one hundred virulent tubercle bacilli resulted in the establishment of recognizable tuberculous foci. Guinea pigs, on the other hand, can and should be tuberculin tested before use to rule out the presence of spontaneous disease.

Another serious criticism of the general adaptation of mice for tuberculosis diagnostic work results from the finding by certain investigators[7] that among human strains of tubercle bacilli there are encountered some which are not of equal virulence for both guinea pig and mouse. Until it can be demonstrated,

through extensive and painstaking investigation, that there exists an exact parallel between virulence for mice and virulence for man, it is doubtful that mouse infection tests will gain general recognition. The objection might be raised, too, from observations made in this laboratory and in others,[11] that the inoculation of mice with virulent tubercle bacilli is not always followed by a constant and predictable degree of infection among animals receiving identical dosages of the same organism.

It was the purpose of the present study to (a) verify some of the more practical laboratory findings concerned with mouse infection tests, and (b) to establish criteria for evaluating the reliability of such tests, some of which will be discussed here briefly.

That different strains of mice vary markedly in their susceptibility to induced tuberculosis has been observed by Dubos and co-workers[16] and it was on the basis of their findings that pigmented (C57 and dba) rather than white animals were used exclusively in the experimental work reported herein. As noted by the Rockefeller group,[16] however, "It is probable that unrecognized variations in the culture, the season of the year, and other uncontrolled factors have an appreciable influence on the course and outcome of the experimental infection." In addition, it is probable that individual mice of the same strain differ from others in the group as a result of certain as yet unknown factors. These might make mandatory a more thorough investigation of identical strains of mice obtained from different breeders. Pending the completion of such studies it would perhaps be wise for each laboratory to maintain a constant and reliable source of mice to eliminate the necessity of re-evaluating new and untried groups of animals.

Another source of variation is the enhancing effect, on tuberculous infections, of a deficient (essentially corn meal and water) diet which has been demonstrated for albino and pigmented mice[17] as well as rats.[31] This experimental evidence was convincing enough to warrant the inclusion of such diets in studies carried on by this laboratory in order to increase susceptibility of our mice to the test infections.

With respect to the mode of inoculation, the intracerebral rather than the intravenous technique was preferred here because of its simplicity and the relative ease with which it can be mastered by the average technician.

The size and time relationships of dosage, all too frequently lost sight of in tuberculosis diagnostic work, should be given careful consideration in mouse infection tests. On the basis of investigations conducted here and in other laboratories, a dosage of 0.01 mg. has been arbitrarily adopted for use with cultures of tubercle bacilli. It was felt that the ideal dosage should be one which facilitates an early (one to three weeks) detection of proliferation in the case of pathogens without being so overwhelming as to produce false positives in the case of saprophytes, a phenomenon which, though yet to be demonstrated with mice, is not unknown in guinea pigs. It can be seen from the experimental results presented that tubercle bacilli can be detected in the various organs by smear even though gross evidence of their presence is lacking. This facilitates

early diagnosis. However, gross pulmonary lesions should be examined microscopically in every instance in order to exclude confusion due to spontaneous diseases closely simulating tuberculosis. Such conditions have been demonstrated in rats.[32] In addition, in two instances in our laboratory pulmonary lesions indistinguishable macroscopically from tubercles have been observed in mice at autopsy in which a diligent search failed to reveal the presence of acid-fast bacilli.

Finally, in regard to the minimum number of animals indicated for valid results, it is recommended that at least four mice be inoculated with each culture to compensate not only for inconstant degrees of tuberculous infection among the animals but also for deaths due to intercurrent infections.

SUMMARY

The susceptibility of pigmented strains of mice (dba and C57) to tuberculosis induced by the intracerebral and intravenous inoculation of virulent human tubercle bacilli has been confirmed.

In these studies, tubercle bacilli of the human, bovine, and avian types were of similar virulence for mice following inoculation by the intracerebral route. An acid-fast saprophyte failed to produce a progressive disease.

In a comparative study involving a series of twenty-five strains of acid-fast bacilli there appeared to be a high degree of correlation between virulence for mice and virulence as measured by classical guinea pig tests.

Preliminary studies indicated that a combination of rapid, diffuse growth in a liquid medium of the Dubos type plus subsequent mouse inoculation would reduce considerably the time required for the laboratory diagnosis of tuberculosis. Penicillin, added to a Tween-albumin medium in a concentration of 2 units per milliliter with the purpose of suppressing the growth of contaminants, proved to be toxic to the tubercle bacilli contained in experimental inocula consisting of digested and neutralized sputum specimens.

Both treated (NaOH) and untreated routine clinical specimens were shown to be extremely toxic when inoculated (intracerebrally) directly into mice in an attempt to recover tubercle bacilli; therefore the routine use of mice as a primary diagnostic procedure is not practical by the methods described.

The theoretical and practical aspects of mouse infection tests are discussed in some detail.

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THE SEROLOGIC RELATIONSHIP OF FUNGUS ANTIGENS

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THE reported concurrence of pulmonary calcification and skin sensitivity to histoplasmin[1-5] has led to the suggestion that infection with *Histoplasma capsulatum* is frequently nonfatal. This depends on the theory that sensitivity to histoplasmin results from a past or present infection with *H. capsulatum*. Emmons and co-workers[6, 7] have demonstrated, however, that a positive skin reaction to histoplasmin may appear in animals experimentally infected with either *H. capsulatum, Blastomyces dermatitidis, Coccidioides immitis,* or *Candida albicans.* Howell[8] demonstrated by skin tests the extensive cross reaction between histoplasmin and blastomycin in guinea pigs infected with *H. capsulatum* and *B. dermatitidis.*

Several workers started serologic investigations with the hope that the information therefrom would aid in the diagnosis of a mild or severe form of histoplasmosis. I[9] described the use of the whole yeastlike cell as a complement fixation antigen, and found it more specific than histoplasmin. Tenenberg and Howell[10] found that both histoplasmin and blastomycin, when used as complement fixation antigens, produced positive reactions in the presence of sera from animals experimentally infected with *B. dermatitidis* or *H. capsulatum.* Fixation was stronger with the homologous than with the heterologous antigen. Saslaw and Campbell,[11] using the sera of rabbits hyperimmunized with heat-killed antigens, found cross reactions between the yeastlike phase of *H. capsulatum* as antigen and anti-*B. dermatitidis* serum, but more especially between the yeastlike phase of *B. dermatitidis* and anti-*H. capsulatum* serum.

With the indication that antigens from the yeastlike phase appeared to be more specific than those from the mycelium, but with little knowledge of the extent of cross reactions between fungus antigens and of the identity of factors in the various antigens, further study of the serologic relationship between fungus antigens seemed desirable. Accordingly, experiments were initiated to determine by complement fixation methods the specificity of and relationships between antigens from *H. capsulatum, B. dermatitidis, C. immitis, C. albicans, Aspergillus fumigatus,* and *Mucor corymbifer.* The first four fungi were chosen for study since Emmons and associates[6, 7] had already established their positive immunologic relationship, in that guinea pigs infected with one of them were capable of showing positive skin reactions to histoplasmin. The last two were included for study because, although pathogenic, they had not been shown as having any relationship to *H. capsulatum* by skin test and because they were so decidedly different morphologically from *H. capsulatum.*

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MATERIALS AND METHODS

Complement Fixation Test.—The complement fixation test, originally described by Bengston,[12] was similar to the one reported in a previous paper.[9]

Fungi.—One strain of each of the six fungi was employed in these studies. Strain 6521 of *H. capsulatum* originally was isolated from a dog[13]; strain 6046 of *B. dermatitidis,* from a human patient in Alabama; strain 6210 of *C. immitis,* from a human patient in Arizona; strain 3148 of *C. albicans,* from a human patient with endocarditis; strain 5225 of *A. fumigatus,* from a penguin; and strain 217 of *M. corymbifer,* from a pea. The antigens from *H. capsulatum, B. dermatitidis,* and *C. albicans* were whole yeastlike cells, which had been killed by suspension in 0.5 per cent formalin for eighteen hours, washed in saline, centrifuged, and then stored in a concentrated form at 2 to 5° C. in 1:10,000 Merthiolate. Since the sporangial phase of *C. immitis* has not been grown extensively in culture, the broth filtrate from mycelial growth (i.e., coccidioidin) was used as the antigen.* Washed spores served as the antigens for *A. fumigatus* and *M. corymbifer. H. capsulatum* was grown in the yeastlike phase in a semifluid peptone medium[14]; *B. dermatitidis,* in a semifluid medium containing a mixture of amino acids plus serine[15]; *C. albicans,* in Sabouraud's dextrose broth; and *A. fumigatus* and *M. corymbifer,* on Sabouraud's dextrose agar. The antigens were assayed against homologous immune sera at irregular intervals to check their binding power and anticomplementary action.

Antisera.—Forty adult albino rabbits were inoculated (a) intravenously with living yeastlike cells (e.g., ten rabbits with *H. capsulatum* and ten with *B. dermatitidis*); (b) intraperitoneally with a suspension of live conidia (e.g., fourteen rabbits with *C. immitis*); or (c) intravenously with increasing dosages of formalin-killed yeast cells (e.g., six rabbits with *C. albicans*). The rabbits were not inoculated with live cells of *C. albicans* because infection with this agent produced rapid death in such animals. The groups of rabbits that were inoculated with one of the live strains were bled once a week thereafter until the termination of the experiment. The sera that were used for the absorption studies were pools from these and other similar bleedings. Only those sera with positive titers with the homologous antigens were included in these pools. Attempts to produce hyperimmune anti-*A. fumigatus* and anti-*M. corymbifer* sera of significantly high titer were unsuccessful, with the result that these two types of sera were not used in the absorption tests in the present study.

EXPERIMENTS AND RESULTS

Individual sera from thirty-four rabbits experimentally infected with *B. dermatitidis, C. immitis,* or *H. capsulatum* and bled once a week were titered against their homologous antigens. Those sera which fixed complement in the presence of the homologous antigen were tested against the three heterologous antigens (Figs. 1 to 3). The *B. dermatitidis* antigen induced the formation of antisera only 8 per cent of which (seven of ninety-two sera) fixed complement with the heterologous *H. capsulatum* antigen and none of which reacted with the *C. albicans* or *C. immitis* antigens. The titers with the *H. capsulatum* antigen against the anti-*B. dermatitidis* serum were noticeably lower than those with the *B. dermatitidis* antigen (Fig. 1). However, *B. dermatitidis* was weaker as an antibody-producing antigen than the other antigens, as evidenced by the fact that its antisera had much lower titers than the other antisera, when each was tested against its homologous antigen (Figs. 1 to 3). The *H. capsulatum* antigen induced antisera which fixed complement in equal quantity with the homologous and the *B. dermatitidis* antigens, although the antibody titers with the latter were slightly lower (Fig. 2). None of the anti-*H. capsulatum* sera reacted with the antigens from *C. albicans* and *C. immitis.* The

*This antigen was obtained from Dr. C. E. Smith, Stanford University, School of Medicine, San Francisco, Calif.

C. immitis-infected animals produced antisera which reacted not only with the homologous antigen but 63 per cent of which also reacted with the *B. dermatitidis* antigen and 6 per cent with the *H. capsulatum* antigen. The antibody titers with *B. dermatitidis* and especially with *H. capsulatum* antigens were

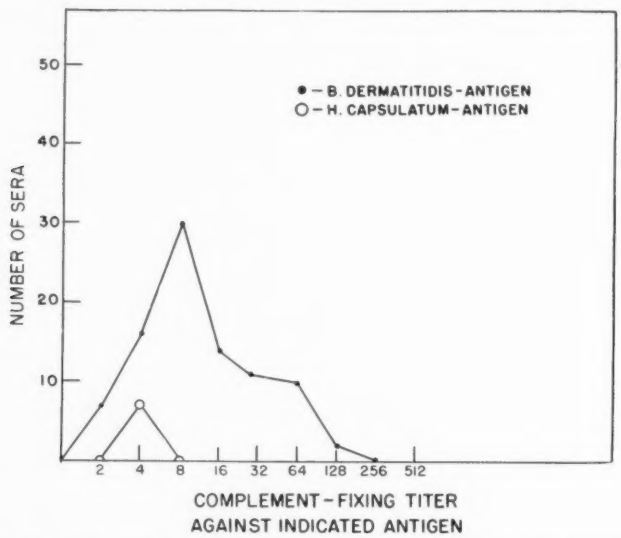

Fig. 1.—Reactivity of anti-*B. dermatitidis* sera with homologous and heterologous antigens. Note: No positive titers were produced with *C. immitis* and *C. albicans* antigens.

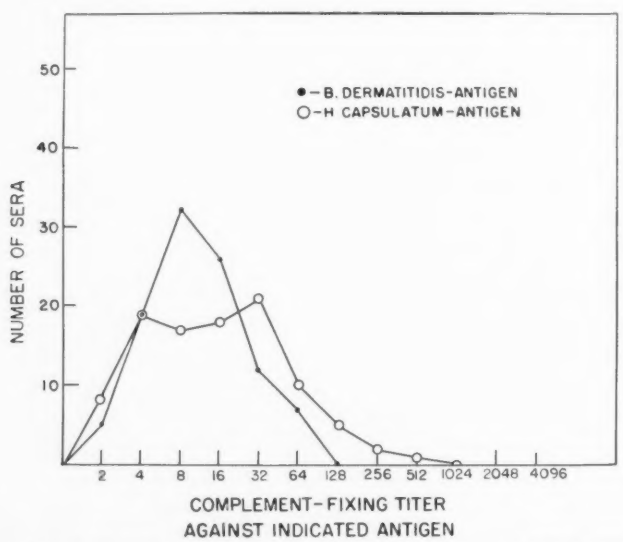

Fig. 2.—Reactivity of anti-*H. capsulatum* sera with homologous and heterologous antigens. Note: No positive titers were obtained with *C. albicans* and *C. immitis* antigens.

much lower than with the homologous antigen. No fixation occurred in the presence of the *C. albicans* antigen. The pooled serum from the rabbits hyperimmunized with the *C. albicans* antigen had an antibody titer of 1:256 with the homologous antigen, of 1:16 with the *H. capsulatum* antigen, and of 1:2 with the *B. dermatitidis* antigen. No titer was obtained with the *C. immitis* antigen.

Thus, *B. dermatitidis* antigen may be considered as forming the most specific antibody (of the four tested), although its complement-fixing antigen was the least specific. *H. capsulatum* induced the formation of a slightly less specific antibody than *B. dermatitidis,* while *C. albicans* and *C. immitis* produced antisera that cross reacted to the greatest extent with heterologous antigens. It may be noted here that the serum titers were relatively low even with the homologous antigens, a characteristic common to most fungus antisera.

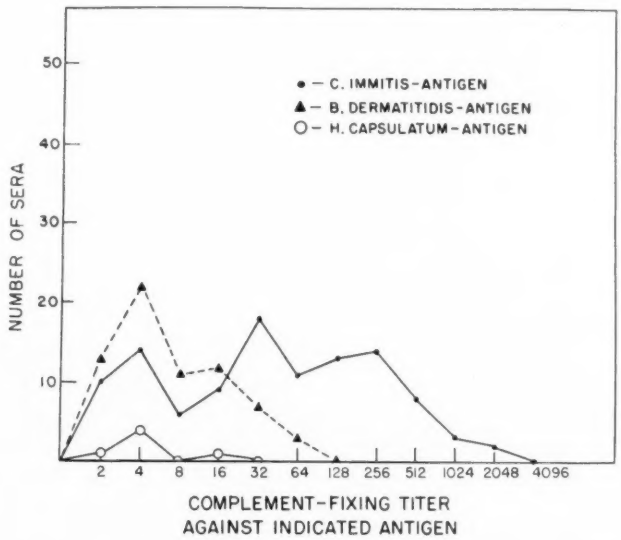

Fig. 3.—Reactivity of anti-*C. immitis* sera with homologous and heterologous antigens. Note: No positive titers were obtained with the *C. albicans* antigens.

Further information on the interrelationship of the fungal antigens was sought in studies involving absorption of antisera with both homologous and heterologous antigens. Pooled rabbit antisera were absorbed with an antigen from one of six fungal species, namely, *H. capsulatum, B. dermatitidis, C. albicans, C. immitis, A. fumigatus,* and *M. corymbifer.* The antigens from the first three species were whole yeastlike cells killed by 0.5 per cent formalin. In the case of *C. immitis,* the antigen used for absorbing antibody was mycelium grown for three weeks at 25° C., killed in 0.5 per cent formalin, washed in saline, and ground thoroughly in a ball mill. The antigens from *A. fumigatus* and *M. corymbifer* were spores washed off agar slants, rewashed in saline, killed in 0.5 per cent formalin, then washed and resuspended in saline. The serum was absorbed with homologous antigen until either no serum titer remained or additional absorption produced no change in titer. Twice the amount of antigen used in the foregoing experiments was employed in the absorption of heterologous antisera. In the tests involving *A. fumigatus* and *M. corymbifer,* the sera were absorbed and reabsorbed until they started to become anticomplementary. The experiments were repeated three times with different sera, with similar results obtained except where indicated.

When the anti-*B. dermatitidis* serum was absorbed with its homologous antigen (Table I), a similar decrease in titer of the serum was shown with both the *B. dermatitidis* and the *H. capsulatum* complement-fixing antigens. This result indicates that the *B. dermatitidis* antigen induces the formation of one antibody or one group of antibodies which is completely responsible for the serologic reaction between anti-*B. dermatitidis* serum and *H. capsulatum* antigen. Absorption with the *C. albicans, C. immitis,* or *H. capsulatum* antigen resulted in partial removal of reactivity with the homologous *B. dermatitidis*

TABLE I. INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION TITERS OF RABBIT ANTISERA

	ANTI-B. DERMATITIDIS SERUM						
		ABSORBED WITH					
ANTIGEN	UNABSORBED	B. DERMATITIDIS	H. CAPSULATUM	C. ALBICANS	C. IMMITIS	A. FUMIGATUS	M. CORYMBIFER
B. dermatitidis	32	4*	4	4	16	32	16†
H. capsulatum	16	4	0	4	0	16	16
C. albicans	0‡	–	–	–	–	–	–
C. immitis	0	–	–	–	–	–	–

*Further absorption produced no decrease in titer.

†The figures are the inverse of the maximum serum dilution that completely fixed complement in the presence of 4 units of antigen.

‡Throughout this paper, "0" indicates no fixation in a serum dilution of 1:2.

complement fixation antigen and almost complete removal with the *H. capsulatum* complement-fixing antigen. The *C. albicans* antigen seemed to have relatively strong absorptive qualities for the anti-*B. dermatitidis* serum, especially when it is noted that this antigen did not fix complement with this serum. Apparently there was not sufficient *C. albicans* antigen in the serologic test to produce a reaction in the presence of the heterologous antiserum. Thus, the antibodies induced by the *B. dermatitidis* antigen appear to contain a factor or factors which are capable of combining in varying degrees, depending on the species, with the preceding three heterologous antigens. *A. fumigatus* and *M. corymbifer* absorbed no significant amount of antibody from the *anti-B. dermatitidis* serum, and therefore probably have no antigenic similarity with *B. dermatitidis.*

Although anti-*C. albicans* serum fixed complement in the presence of *B. dermatitidis* and *H. capsulatum* antigens in low titers, absorption with the homologous *C. albicans* antigen removed the cross reactions with these two heterologous antigens (Table II). Thus, the *C. albicans* antigen contains a factor that is the same in serologic reaction as the one in *B. dermatitidis* and *H. capsulatum* antigens that causes fixation of complement in the presence of anti-*C. albicans* serum. Absorption with either *B. dermatitidis* or *H. capsulatum* antigens resulted in the removal of antibody from anti-*C. albicans* serum, as shown with the *C. albicans, B. dermatitidis,* or *H. capsulatum* complement fixation antigens. When the antiserum was absorbed with *C. immitis* antigen, only part of the antibody was removed. These two observations indicate that the

anti-*C. albicans* serum has a substance that can react well with *B. dermatitidis* and *H. capsulatum* antigens, and to a more limited extent with *C. immitis* antigen. Because of their failure to absorb any antibody from anti-*C. albicans* serum, *A. fumigatus* and *M. corymbifer* antigens were considered unrelated to the *C. albicans* antigen.

TABLE II. INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION TITERS OF RABBIT ANTISERA

	ANTI-C. ALBICANS SERUM						
		ABSORBED WITH					
ANTIGEN	UNABSORBED	B. DERMATITIDIS	H. CAPSULATUM	C. ALBICANS	C. IMMITIS	A. FUMIGATUS	M. CORYMBIFER
C. albicans	256	0	0	0	64	256	256
B. dermatitidis	2	0	2	0	2	2	2
H. capsulatum	16	0	0	0	8	8	16
C. immitis	0	–	–	–	–	–	–

Anti-*C. immitis* serum, after absorption with its homologous antigen, still partly reacted with the *B. dermatitidis* and *H. capsulatum* complement fixation antigens (Table III). Thus, there are nonspecific factors in the anti-*C. immitis* serum which react serologically with the *H. capsulatum* and *B. dermatitidis* antigens, but not with the homologous antigen. Absorption with *B. dermatitidis* and *H. capsulatum* antigens resulted in almost complete loss of antibody capable

TABLE III. INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION OF RABBIT ANTISERA

	ANTI-C. IMMITIS SERUM						
		ABSORBED WITH					
ANTIGEN	UNABSORBED	B. DERMATITIDIS	H. CAPSULATUM	C. ALBICANS	C. IMMITIS	A. FUMIGATUS	M. CORYMBIFER
C. immitis	32	0	0	16	0	32	16
B. dermatitidis	16	0	0	16	8*	16	8
H. capsulatum	16	2	0	16	8*	16	16
C. albicans	0	–	–	–	–	–	–

*These paradoxical residual titers to the heterologous or nonspecific antigens, after the complete removal of the homologous antibodies, may represent a nonspecific complement fixation. The antigen and absorbed serum, both slightly anticomplementary, may remove enough complement to produce a pseudo-complement fixation.

of reacting with either the two foregoing antigens or the *C. immitis* antigen; whereas repeated absorption of the anti-*C. immitis* serum with *C. albicans* antigen produced only a slight decrease in antibody. No absorptive qualities were shown by the antigens from *A. fumigatus* and *M. corymbifer*.

Finally, when the anti-*H. capsulatum* serum was absorbed with the homologous antigen, the titer with *B. dermatitidis* complement fixation antigen was greatly reduced (Table IV). The anti-*H. capsulatum* serum may then be

TABLE IV. INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION TITERS OF RABBIT ANTISERA

ANTIGEN	ANTI-H. CAPSULATUM SERUM						
		ABSORBED WITH					
	UNABSORBED	B. DERMATITIDIS	H. CAPSULATUM	C. ALBICANS	C. IMMITIS	A. FUMIGATUS	M. CORYMBIFER
H. capsulatum	64	0	0	32	32	32	64
B. dermatitidis	16	4	4	8	8	16	16
C. albicans	0	–	–	–	–	–	–
C. immitis	0	–	–	–	–	–	–

considered as containing a factor common to *H. capsulatum* and *B. dermatitidis* antigens. This common factor in the anti-*H. capsulatum* serum is completely responsible for the reaction with the homologous antigen, since absorption with *B. dermatitidis* produced a reduction in antibody equal to that produced by the homologous antigen. *C. albicans* and *C. immitis* antigens reduced titers only partially, whereas *A. fumigatus* and *M. corymbifer* antigens had no effect.

DISCUSSION

The term "antigen" may be referred to (a) as the substance that induces the formation of antibody or (b) as the substance that reacts with antibody in a serologic test. On application of the first definition to the serologically interrelated fungi, *B. dermatitidis*, *C. albicans*, *C. immitis*, and *H. capsulatum*, the antigen from *B. dermatitidis* may be considered the most specific of the four, since it produced an antiserum that reacted only with the homologous antigen and to a much more limited extent with *H. capsulatum* antigen. However, on the application of the second definition, it was the least specific serologic antigen, since it fixed complement not only with its homologous antiserum but also with the three heterologous antisera. Similarly, *H. capsulatum* antigen produced a relatively specific antiserum in that it reacted about equally with its homologous antigen and the *B. dermatitidis* antigen. As a complement fixation antigen, it was conversely nonspecific since it fixed complement with the three heterologous antisera. The antigens of *C. albicans* and *C. immitis* incited the formation of antibodies that reacted with three of the four antigens studied, although as serologic antigens reactions with the homologous antisera alone were obtained.

Since absorption of a serum with a homologous antigen resulted in a reduction of titer with a heterologous antigen, and since each antigen was capable of absorbing at least some of the antibody from each of the heterologous antisera, a common factor or factors must be present in the antigens of the four species. Hence, the antigen from any of these fungi is inherently capable of reacting serologically with any one of the four antisera, although in the complement fixation tests with both pooled and individual rabbit sera the antigens from *C. albicans* and *C. immitis* did not react with heterologous antisera. However, others[16, 17] have reported that the sera of rabbits immunized against *B.*

dermatitidis fixed complement in the presence of *C. albicans antigen*. No indication of a serologic cross reaction between the complement-fixing antigen of *C. immitis* and a heterologous antiserum has been found in the literature. Why in a previous series of experiments[9] cross reactions between anti-*B. dermatitidis* serum and *H. capsulatum* yeastlike cells as antigen were not obtained is in doubt, although the reason may lie in differences in the strains used.

Of interest is the failure of *A. fumigatus* and *M. corymbifer* antigens to absorb significant amounts of antibodies from any of the antisera under study—a characteristic probably due to the antigenic dissimilarity between *A. fumigatus* and *M. corymbifer* on the one hand and *B. dermatitidis, C. albicans, C. immitis,* and *H. capsulatum* on the other. This apparent lack of antigenic relationship is especially striking, since *M. corymbifer* is more closely allied morphologically and taxonomically to *C. immitis* than either *B. dermatitidis, C. albicans,* or *H. capsulatum,* while *A. fumigatus* is closer to the latter three species than *C. immitis.*

The serologic specificity of the four related antigens was paralleled by their antigenic potency—the more specific the antigen, the greater its antibody-inducing powers. Thus, *C. albicans* and *C. immitis* antigens produced the highest titered sera, whereas *B. dermatitidis* antigen induced sera of low titers. This high titer of a serum may partly be the basis for the reaction with heterologous antigens. Frequently, the binding power of the *B. dermatitidis* antigen was about the same or only slightly higher than the anticomplementary action, with the result that four units of the antigen could not be used and the lot had to be discarded.[18]

With each of the antigens of *B. dermatitidis, C. albicans, C. immitis,* and *H. capsulatum* capable of cross reaction with the sera of each other, a positive complement fixation titer in an unknown rabbit serum might theoretically be due to an antibody produced by one of the other fungi. However, the antigens may still be considered to be of value in the differential diagnosis of mycotic disease, when it is emphasized (a) that fixation of complement with a heterologous antigen was generally at very low serum dilutions, i.e., 1:8 and below, and (b) that the titer of a serum was usually distinctly higher with the homologous than with a heterologous antigen. Several sera from an animal with a suspected mycotic disease should be collected and tested over an extended period of time, and if titers are low they should be tested with all four antigens.

SUMMARY AND CONCLUSIONS

Complement fixation methods were employed in the determination of the specificity of and the relationship between antigens from *Aspergillus fumigatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Histoplasma capsulatum,* and *Mucor corymbifer.* Sera from animals experimentally infected or hyperimmunized with one of the pathogenic fungi were absorbed with each of the six fungal antigens. Also, sera from experimentally infected rabbits were examined by complement fixation tests to determine the extent of their reactions with homologous and heterologous antigens.

A. fumigatus and *M. corymbifer* were probably antigenically unrelated to the other four species, since they failed to absorb antibodies from any of the heterologous antisera. Although *B. dermatitidis* antigen was the least specific complement-fixing antigen of the four remaining, it produced the most specific antibody. Its antiserum reacted with the homologous antigen and slightly with the *H. capsulatum* antigen, and was absorbed by the *H. capsulatum* antigen and to a lesser extent by the *C. albicans* and *C. immitis* antigens. *H. capsulatum* antigen produced serum that reacted about equally with homologous antigen and *B. dermatitidis* antigen, and that was absorbed by *B. dermatitidis* antigen and to a lesser extent by the *C. albicans* and *C. immitis* antigens. The latter two antigens produced sera that fixed complement with and were absorbed by two of three heterologous antigens, namely, *B. dermatitidis* and *H. capsulatum* antigens.

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THE ELECTROCARDIOGRAM OF NORMAL AND MALARIA-INFECTED MONKEYS

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PRELIMINARY to the study of comparative effects of intravenous quinine dihydrochloride in normal and malaria-infected monkeys,[1] it was incumbent upon us to establish the variations of the electrocardiogram in these two groups of animals. The available data appeared inadequate. Lloyd[2] noted for seventeen *Macacus rhesus* monkeys averages for the P-R interval of 0.08 sec., the QRS width 0.04 sec., the Q-T length 0.20 sec., and electrocardiographic patterns similar to those of man. DeWaart and Storm[3] published the following norms for some fifteen *Macacus irus* monkeys: P-R intervals from 0.54 to 0.088 sec., QRS widths from 0.02 to 0.044 sec., and the angle of the QRS axis from +50 to +75 degrees. They established the empirical validity of the Einthoven triangle for the monkey as for man and claimed that the P and T waves were always positive and the S-T intervals isoelectric as in man, and not as in the dog. We considered it necessary to establish the electrocardiographic findings in a larger series of normal monkeys, with the addition of the chest lead. No detailed electrocardiographic observations in malaria-infected monkeys were found in the literature.

MATERIALS AND METHODS

In this study twenty-seven normal *Macaca mulatta* monkeys, weighing 2 to 7 kilograms, were strapped on their backs to an animal board. Leads I, II, III, and an apical chest lead (corresponding to IV F) were obtained in the usual manner with a Cambridge electrocardiograph. Repeated tracings were taken in some instances on different days, for a total of thirty-four observations, to determine the variations in single monkeys and to establish the normal electrocardiographic range. Twelve monkeys infected with *Plasmodium knowlesi*, on which normal electrocardiograms were obtained previously, had electrocardiograms with four leads similarly taken on twenty-two occasions during varying stages of the infection. The course of the disease was followed by counting the number of red blood cells and the number of parasitized cells per 500 erythrocytes in the peripheral blood.

EXPERIMENTAL RESULTS

The electrocardiograms of normal monkeys presented heart rates varying from 170 to 280 beats per minute, P-R intervals from 0.06 to 0.10 sec., QRS intervals from 0.02 to 0.05 sec., Q-T intervals from 0.14 to 0.20 sec., the QRS axis angle $\propto$ from +50 to +115 degrees. A single auricular ectopic beat was the only arrhythmia noted. We found (Table I) a flat T_1 in nine of thirty-four observations, a negative T_1 in one instance, flat T_2 in three, negative T_2 in three, flat T_4 in two, and negative T_4 in two instances. S-T depression, particularly in Leads II and III, of 1 mm. or more was noted in eight of twenty-seven monkeys. In two monkeys a negative and flat T_1 was found on different

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TABLE I. ELECTROCARDIOGRAPHIC DEVIATIONS FROM THE AVERAGE IN NORMAL AND MALARIA-INFECTED MONKEYS

	T_1 FLAT	T_1 NEG.	T_2 FLAT	T_2 NEG.	T_4 FLAT	T_4 NEG.	S-T DEPRESS	TOTAL CASES	TOTAL ECG
Normal	9	1	3	3	2	2	8	27	34
Malaria	6	3	1	1	0	0	3	12	22

days; in two other instances negative and flat T_2 waves were similarly noted; in one other instance the T_4 was flat and negative on two different occasions.

Malaria-infected monkeys presented heart rates in the same range as the normal monkeys with the exception of one monkey with a rate of 159 beats per minute. The atrioventricular and intraventricular conduction times had the same limits as in the normal animals. The Q-T intervals (electrical systoles) were in the upper range of normal controls; in only one severely infected monkey was a Q-T interval of 0.23 sec. present, with a heart rate of 159 beats per minute. Two animals, one with moderate and one with severe parasitemia and anemia, showed marked left axis deviations, –40 and –48 degrees. In all others the axes were within the normal range. No arrhythmias were noted in the malaria-infected animals.

From Table I it will be seen that, in general, the proportion of negative T waves in the malaria-infected monkeys was the same as in the normal controls; nor were these negative T waves and depressed S-T segments in the malaria-infected monkeys present particularly in the more severely infected animals. In two of three animals presenting negative T_1 waves they were flat on other occasions, and the same variations were seen in these two monkeys before their malarial infection (Fig. 1).

DISCUSSION

It is clear from our observations of the electrocardiogram in twenty-seven normal *Macaca mulatta* monkeys, which is the largest group so far published, that the variability, particularly in the recovery portion (ST-T segment) of the normal tracing, is much greater than heretofore realized. Hence, if one followed the human standards of electrocardiographic criteria for the monkey, many of our findings in the malaria-infected cases would be classed as abnormal. On the other hand, comparing their tracings with the ones obtained before the infection with *P. knowlesi*, one comes to the conclusion, as did Sprague[4] in thousands of cases of human malaria, that the electrocardiogram is unchanged in the vast majority of instances.

Three electrocardiographic observations in malaria-infected monkeys revealed deviations from the normal variants. Two of these involved leftward changes in the axis which are difficult to interpret. The Q-T interval tended to be in the upper ranges of normal, and in one instance was somewhat prolonged, even considering the relatively slow heart rate. This might indicate the mildest form of myocardial malfunction or fatigue, which may be so expressed under other circumstances. The failure to show significant electrocardiographic abnormalities in malaria-infected monkeys is thoroughly consistent with the absence of specific pathologic lesions in the hearts of malaria-infected monkeys[5] and of human beings with malaria.[6]

In some instances of severe anemia there are seen electrocardiographic changes suggestive of coronary insufficiency and nonspecific myocardial damage, presumably correlated with anoxemia, fatty degeneration, and even dilatation of the heart, all of which may occur in severe malarial infection.[5] The S-T and T segment variations in our malaria-infected monkeys, however, did not

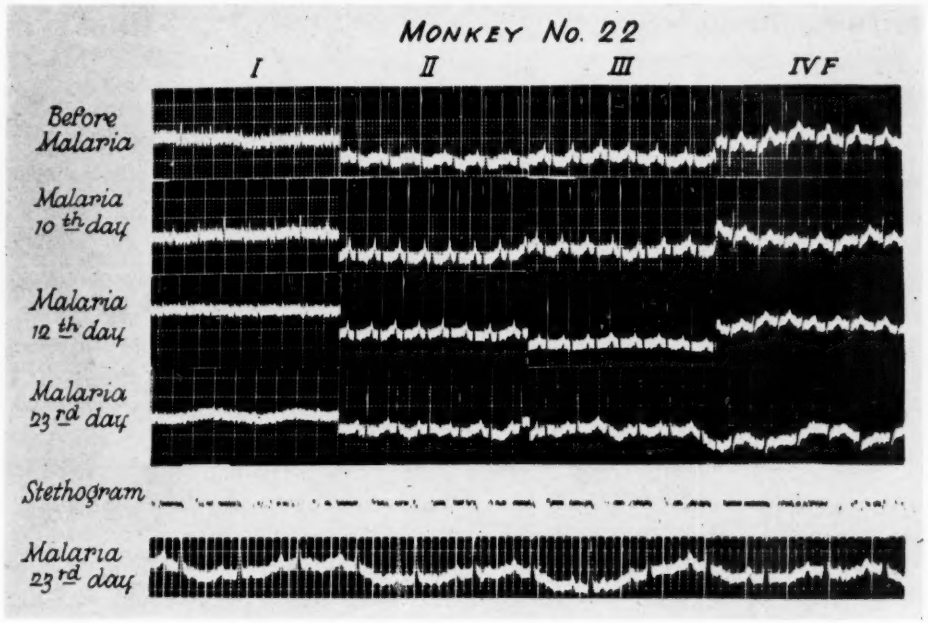

Fig. 1.—Before malaria the T_1 wave was flat, and S-T_2 and S-T_3 segments were depressed in this monkey; T_2 was either negative or diphasic on different occasions. With red blood counts of 5.88, 3.6, and 1.58 on the tenth, twelfth, and twenty-third day (day before death) of malarial infection respectively, the electrocardiogram was not significantly changed except for the variations in T_2, as noted also before malaria, and progressive prolongation of the Q-T interval from 0.16 to 0.18 seconds. Stethogram on the day before death was normal.

differ significantly from those of the controls. Nor did we see any prolongation of the P-R interval. Our lowest attained red blood cell count, 1.6 million per cubic millimeter, may not have been low enough to cause significant anoxemic electrocardiographic changes. In several instances, stethographic (Fig. 1), fluoroscopic, and roentgenographic examinations of the heart showed no murmurs, gallop rhythm, or significant cardiac dilatation in severe and even fatal cases of malaria.

SUMMARY AND CONCLUSIONS

1. The normal electrocardiogram of the *Macaca mulatta* monkey is between that of the dog and man. The variability includes negativity of the T waves in Leads I, II, and IV F and displacement of the S-T segments from the isoelectric line.

2. The electrocardiogram of malaria-infected *Macaca mulatta* monkeys does not differ from that of the controls in the great majority of instances. The

rare exceptions include marked (left) axis deviation and slight prolongation of the Q-T interval. No typically anoxemic electrocardiographic changes were demonstrated, even in monkeys showing red blood cell counts only 25 per cent of normal.

3. The absence of significant electrocardiographic alterations in monkeys infected with *P. knowlesi* is consistent with the known pathologic changes in malaria in monkeys and with the findings of others in a large series of cases of malaria in human beings.

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LETHAL EFFECTS AND ELECTROCARDIOGRAPHIC CHANGES PRODUCED BY QUININE DIHYDROCHLORIDE IN MALARIA-INFECTED MONKEYS

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INTEREST in the lethal effects of quinine on monkeys infected with malaria developed recently when it was observed that some animals died following an intravenous injection of quinine, while the same amount had no noticeable effect on other monkeys.[1] This observation brought to mind the controversy of fifty or sixty years ago as to whether the mortality rate was greater in cases of severe malarial fever treated with or without quinine. Deaderick[2] stated that the mortality rate was 25.9 per cent in 1,821 cases of blackwater fever treated with quinine, while it was only 11.1 per cent in 1,006 similar cases not treated with quinine. Several workers have warned against the intravenous injection of quinine in human beings. Brahmachari[3] advised that in all cases of malarial fever in which the blood pressure was low, quinine dihydrochloride, when given intravenously, should be injected slowly, and accompanied by either pituitrin or adrenalin. Maxey[4] warned that if the patient is already suffering from circulatory embarrassment, or the severe hypotonia sometimes associated with chronic estivo-autumnal infections, the added burden produced by the well-intended therapeutic effort may result in a fatal outcome instead of improvement.

Large doses of quinine given intravenously to human beings directly depress the heart and lower the blood pressure.[5] The disturbance in heart action seems to reach its maximum in somewhat less than two minutes, as 95 per cent of an intravenous dose of quinine is removed from the blood stream within five minutes.[6] Respiration and pulse rates are stimulated by small doses and depressed by larger ones. Death occurs usually in coma from cessation of respiration.[5]

In this experimental study observations were made, first, to determine whether quinine dihydrochloride is fatal in smaller doses when given to monkeys with a severe malarial infection than when given to normal monkeys, and, second, to determine the electrocardiographic effects produced by quinine in these two groups of animals.

MATERIALS AND METHODS

Thirteen normal *Macaca mulatta* monkeys, weighing from 2 to 7 kilograms, were injected intravenously with doses of quinine dihydrochloride varying from 20.6 to 82.8 mg. per kilogram. A total of nineteen injections of quinine was given to these monkeys. Eleven animals infected with malaria were given quinine dihydrochloride in doses varying from 18.7 to 41.3 mg. per kilogram. A total of twenty-one injections of quinine was given to these monkeys.

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The quinine was always given intravenously over a period of thirty to forty seconds. The preparation contained 0.5 Gm. of quinine dihydrochloride in each cubic centimeter, or 0.477 Gm. of quinine in combination.

Plasmodium knowlesi was used to infect these monkeys. The course of the disease was followed by red blood cell counts and by counting the number of parasitized red cells per 500 erythrocytes in the peripheral blood. Electrocardiographic tracings were taken at varying times during the infection.

The electrocardiograms were obtained with the monkeys on their backs with each extremity tied to an animal board. Leads I, II, III, and an apical chest lead (corresponding to IV F) were made. A series of tracings was taken for the control, and during the time of the injection of the quinine Lead II was recorded. Four more sets of tracings were obtained immediately, two, four, and ten minutes later. The total number of tracings made for this study, including the normal controls, was 215.

RESULTS

Table I shows the effect of different amounts of quinine dihydrochloride when given intravenously to normal and malaria-infected animals. The maximum amount of quinine that could be given to a normal monkey without producing death was approximately 55 mg. per kilogram body weight. Ten malaria-infected monkeys died with doses of quinine varying from 20.8 to 41.3

TABLE I. AMOUNT OF QUININE DIHYDROCHLORIDE GIVEN INTRAVENOUSLY THAT PRODUCED DEATH IN NORMAL AND MALARIA-INFECTED MONKEYS

NORMAL MONKEYS

SURVIVED		DIED	
MONKEY	QUININE (MG./KG.)	MONKEY	QUININE (MG./KG.)
19	20.6	33	55.5
20	21.3	1	62.5
22	22.7	3	82.8
17	23.9		
11	25.9		
2	31.3		
32	42.5		
35	45.4		
34	45.4		
33	45.7		
31	50.0		
1	55.2		
3	55.2		

MALARIA-INFECTED MONKEYS

SURVIVED				DIED			
MONKEY	QUININE (MG./KG.)	R.B.C. (MILLIONS)	PARASITEMIA PER 500 R.B.C.	MONKEY	QUININE (MG./KG.)	R.B.C. (MILLIONS)	PARASITEMIA PER 500 R.B.C.
21	18.7	3.1	14	8	20.8	3.04	265
13*	20.6	3.46	69	21	22.7	2.04	36
19	20.6	6.48	62	22	22.7	1.66	24
22	22.7	3.6	12	7*	27.5	1.9	237
22	22.7	1.58	37	13	27.5	1.9	161
5	26.9	–	62	28*	27.5	1.21	122
28*	27.0	3.39	3	10	31.4	3.24	154
7	27.5	4.1	127	30*	35.5	–	9
30*	28.4	–	16	11	37.5	–	121
12	37.5	–	0	16	41.3	–	51
16*	41.3	–	14				

*No electrocardiographic record.

mg. per kilogram body weight. Six of these ten animals died following the injection of 27.5 mg. or less of quinine per kilogram of body weight. The malaria-infected monkeys that succumbed to the quinine had severe anemia and usually a high degree of parasitemia. Ten monkeys with less severe anemia and fewer parasites in the circulating blood survived a dose of quinine of equal size (Table I).

TABLE II. LETHAL EFFECT OF QUININE DIHYDROCHLORIDE ON MONKEYS INFECTED WITH P. KNOWLESI

MONKEY	WEIGHT (KG.)	EXPERIMENTAL DAY	R.B.C. (MILLIONS)	PARASITEMIA PER 500 R.B.C.	QUININE (MG.)	RESULTS
21	4.0	10	5.8	10	75	Survived
		12	4.2	14	75	Survived
		13	3.1	14	75	Survived
	3.3	29	2.04	36	75	Died immediately
22	3.3	10	5.88	89	75	Survived
		12	3.6	12	75	Survived
		22	1.62	35	75	Survived
		23	1.58	37	75	Survived
		24	1.66	24	75	Died 30 min. later
375	-	9	3.58	129	75	Survived
		21	1.29	45	75	Died immediately
7	3.63	10	4.1	127	100	Survived
		12	1.90	237	100	Died immediately
28		2	-	3	100	Survived
		3	-	10	100	Survived
		4	-	46	100	Survived
	3.7	6	3.39	3	100	Survived
	3.18	11	1.21	122	87	Died immediately
16	3.63	6	-	14	150	Survived
		7	-	1	150	Survived
		12	-	51	150	Died immediately

Six monkeys with malaria were given the same amount of quinine intravenously one to four times preceding the last injection of the identical amount of quinine which resulted in their immediate deaths (Table II). These monkeys lost some weight during their disease; however, this factor alone apparently cannot account for their response to the same quantity of quinine at different intervals. Monkey 28 survived 100 mg. (27.4 mg. per kilogram) of quinine on four different occasions and died following the fifth injection of 87 mg. (27.1 mg. per kilogram). The anemia was severe at the time of death in each of these six monkeys. Fig. 1 shows the correlation between the anemia, parasitemia, and the periodic intravenous injections of quinine in Monkey 7.

The monkeys given the larger amounts of quinine first showed respiratory difficulties beginning about sixty seconds following the time of the injection. Respirations markedly decreased and frequently stopped for varying intervals, following which time the monkeys gasped irregularly. During this period cyanosis and slowing of the heart occurred, and in such animals death usually followed in two to ten minutes. With a slightly smaller dose of quinine the monkeys developed severe convulsions, preceded and followed by twitchings of different groups of muscles. Cyanosis was frequently marked during the apnea and convulsions. Some of the monkeys that had severe convulsions died

five to fifteen minutes later. The animals that survived the convulsions were unable to stand when returned to their cages thirty to sixty minutes following the injection of the quinine. Gradually these monkeys improved and twelve to eighteen hours later appeared normal. With the smaller doses of quinine no clinical changes were observed.

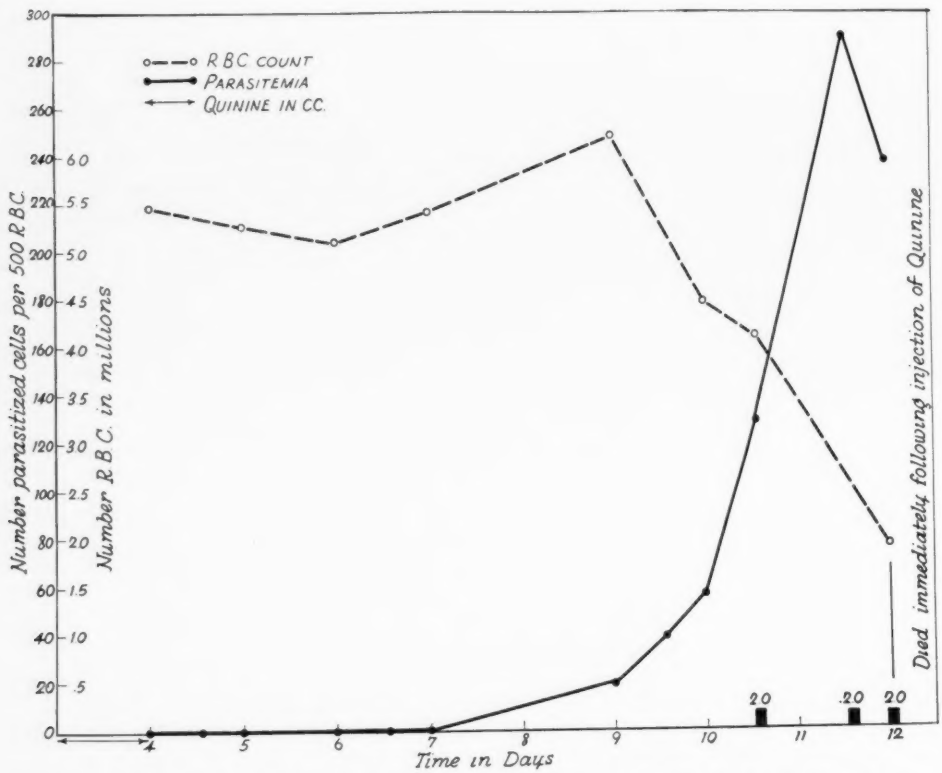

Fig. 1.—Monkey 7. There is a progressive and rapid increase in the degree of parasitemia and a corresponding decrease in the number of red cells in the typical case of malarial infection in the monkey. Death occurred immediately following the third intravenous injection of 2 c.c. of quinine. Death following the injection of quinine may be correlated with the anemia.

The electrocardiographic effects of quinine given intravenously in both normal and malaria-infected monkeys were usually maximum immediately after the completion of the injection. All the intervals usually returned to normal in six to thirty minutes, with the exception of the Q-T interval. This was prolonged for indefinite periods of time following larger doses of quinine in both normal and malaria-infected monkeys. Not infrequently, however, the Q-T interval was 0.1 to 0.2 seconds shorter than the control duration at ten minutes after the injection of small doses of quinine in normal monkeys (Fig. 2). S-T segment deviations and T-wave reversals occurred after quinine in both normal and malaria-infected animals.

The effects of near-equivalent doses of quinine in three representative normal and subsequently malaria-infected monkeys are shown in Table III. In

it are compared in per cent the maximum prolongations of the arterioventricular and intraventricular conduction times and of the electrical systole, and the decreases in the heart rate. In general, it may be said that the same doses of

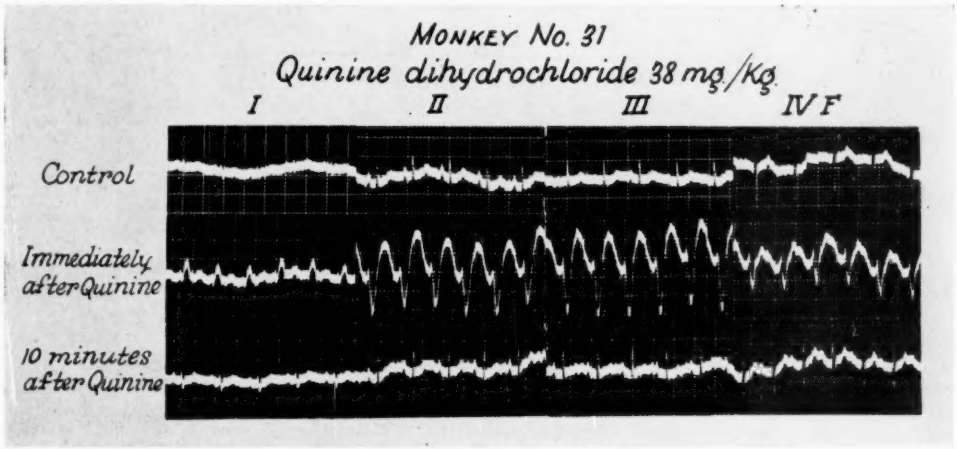

Fig. 2.—Monkey 31. Reversible electrocardiographic effects of a fairly large dose of intravenous quinine in a normal monkey. Prolongation of the P-R, QRS, and Q-T intervals, with a marked left axis shift. Ten minutes following the quinine the atrioventricular conduction time was still slightly prolonged, the intraventricular conduction time normal, and the Q-T interval actually shorter than in the control.

quinine cause more marked effects on these electrocardiographic findings when the monkeys are infected than when they are normal. Furthermore, the more anemic the animals, the greater are the effects produced by similar doses of quinine.

Table III. Typical Cardiodepressive Effects of Equivalent Doses of Quinine Dihydrochloride in Same Normal and Malaria-Infected Monkeys

Monkey	Parasitized R.B.C. per 500	R.B.C. (millions)	Quinine dihydrochloride (mg./kg.)	Prolongation (%)			Decreased heart rate (%)	Remarks
				P-R	QRS	Q-T		
21	0	—	18.8	25	120	12.5	11.0	
21	10	5.8	18.8	54	100	12.5	8.0	
21	14	4.2	20(?)	33	100	12.5	8.5	
21	14	3.1	21(?)	36	160	18.0	11.0	
21	36	2.0	22.7	160+	300+	46.0+	44.0+	Died
19	0	—	21.2	33	133	12.5	0.0	
19	62	6.48	21.2	39	140	19.0	12.0	
19	192	3.76	21.2	70	133	25.0	53.0	Died in 18 hours
22	0	—	22.7	29	80	30.0	0.0	
22	89	5.9	22.7	43	167	25.0	27.0	
22	12	3.6	22.7	43	233	31.0	4.0	
22	35	1.6	22.7	100	471	60.0	55.5	
22	37	1.6	22.7	111	375	83.0	27.0	Barely recovered
22	24	1.6	22.7	178+	400+	120.0+	69.0+	Died

The effects on the three electrocardiographic intervals of the various doses of quinine used in both the normal and malaria-infected animals respectively are plotted in Fig. 3. By contrasting the two groups, a graphic illustration is provided to show that smaller doses of quinine produce greater cardiodepressive effects in malaria-infected monkeys than in normal animals. These electro-

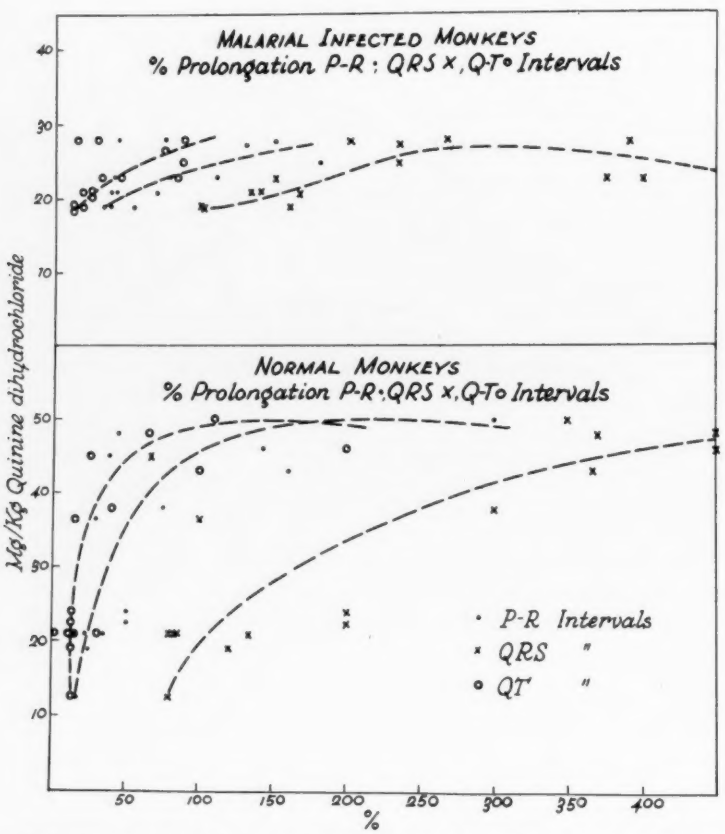

Fig 3.—The variation of the prolongation by varying doses of quinine of the atrioventricular and ventricular conduction times and the Q-T interval in normal and malarial monkeys. Much larger doses of quinine usually failed to prolong the electrocardiographic intervals in normal monkeys than smaller doses did in malaria-infected animals.

cardiographic findings thus corroborate the clinically observed effects on the respiratory and neuromuscular systems and the higher mortality in malaria-infected monkeys than in the normal monkeys following equivalent doses of quinine.

It should be emphasized that the electrocardiographic changes induced by quinine in normal and infected monkeys vary only in degree and not in kind. The pattern following large and sublethal doses is as follows: prolongation of the intervals; slowing of the rate; auricular standstill with increasingly slower idioventricular rhythm; not infrequently periods of ventricular tachycardia and ventricular ectopic beats; rarely auricular fibrillation or flutter with varying A-V block. All of these changes are reversible in animals that recover

from these severe cardiac effects, as in those that survive respiratory arrest and convulsions lasting several minutes.

The electrocardiographic findings in monkeys that died were typified by increasing intraventricular block and ventricular slowing and asystole (Fig. 4). Frequently, however, respiratory failure was followed by reversal of the severe

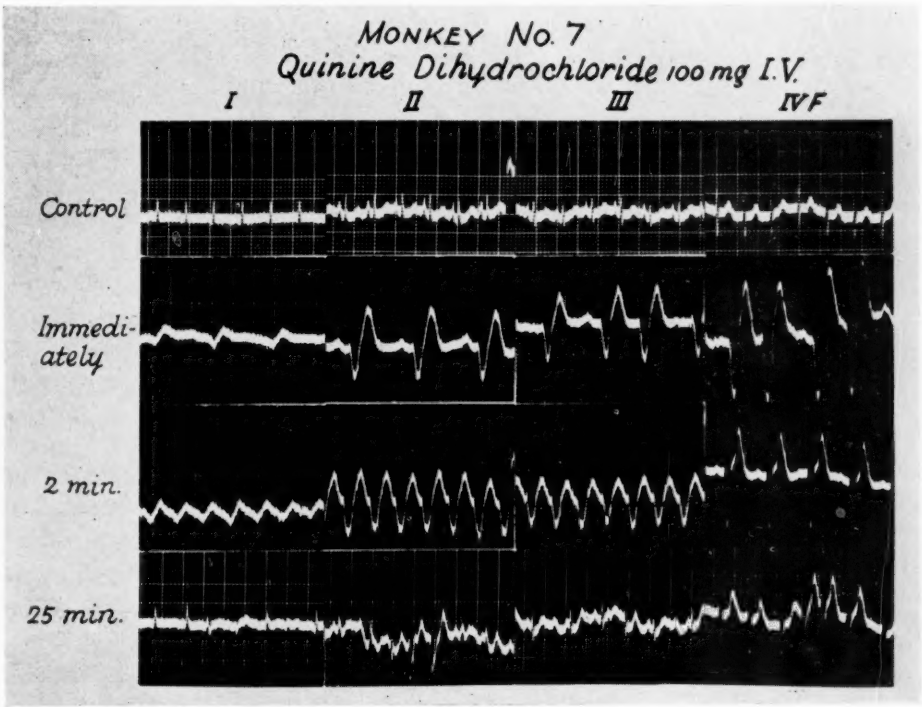

Fig. 4.—Monkey 7. Partially reversible electrocardiographic effects of a relatively small dose (27.7 mg. per kilogram) of quinine in a malaria-infected monkey (parasitemia, 127 per 500 R.B.C.; red blood count, 4.1 million). Marked slowing of the rate; ventricular ectopics preceding and following ventricular tachycardia; reversal of the conduction defects twenty-five minutes after quinine administration.

electrocardiographic manifestations, so that only slow sinus rhythm with fairly short P-R, QRS, and Q-T intervals accompanied or followed by five to twenty minutes "clinical death."

The rapid accession and recession of cardiac effects produced by quinine, also noted by previous observers,[6] were emphasized in our experiments. As an example, nearly identical conduction defects followed small (e.g., 20 mg. per kilogram) doses of quinine, repeated in ten minutes, at which time the effects of the first dose were usually completely gone. Larger (e.g., sublethal) doses required up to thirty or more minutes for complete reversal. Auricular arrhythmias and prolonged Q-T intervals tended to persist following such dosage for the longest period of time. Gallop rhythm was noted stethographically only once in seven observations. Fluoroscopic and roentgenologic studies in a few monkeys revealed no significant enlargement of the cardiac chambers.

DISCUSSION

In this study no attempt was made to establish the minimal lethal dose of quinine, either for normal monkeys or for animals in varying stages of malarial infection. Although the number of animals used in these experiments is too small for statistical purposes, the data would indicate that a monkey with a severe malarial infection is more susceptible to the action of quinine than a monkey with a less severe malarial infection. It would appear from our data that the susceptibility to quinine is related more closely to the anemia than it is to the degree of parasitemia. Of course, the anemia in malaria results from the destruction of the red blood cells by the parasites. In an untreated animal there is a close correlation between the degree of parasitemia and the anemia. However, when quinine is given the parasitemia may decrease without an immediate increase in the number of red blood cells.

It is well known that the salts of quinine frequently are lethal when given intravenously. McCarrison and Cornwall in 1918,[7] while studying the pharmacodynamics of quinine in sheep, showed that "all the salts of quinine employed caused a profound fall of blood pressure not accompanied by a cessation, or even much diminution in the strength, of the heart's beat, except in the two instances in which the respiratory center failed. . . . The fall of blood pressure is usually recovered from in four or five minutes but the period of cardiovascular depression may last for a considerable time." These investigators also emphasied the fact that "the respiratory center is more gravely affected than the cardiac center." Maxey[4] noted in human patients given rapid intravenous injections of quinine symptoms simulating the early findings in our monkeys. Dizziness, sweating, hypotension, dilated pupils, twitchings of the face lasting three to ten minutes, and possible convulsions were, in his experience, danger signals.

The electrocardiographic findings in normal and malaria-infected monkeys have been reported by us in a previous paper.[8] There is little variation from normal in the electrocardiographic pattern of monkeys with a severe malarial infection. The changes in a monkey with a severe malarial infection given a lethal dose of quinine are similar to those in a normal monkey given a lethal dose of quinine, although the quantity of quinine given to the malaria-infected animal is only about one-half that necessary to produce death in the normal monkey. The conduction times and electrical systole are more markedly prolonged in quinine-treated monkeys with a severe malarial infection than in normal animals and, also, those monkeys with a less severe malarial infection. The occasional shortening of the Q-T interval seen ten minutes after the injection of quinine parallels the same finding in isolated rabbit hearts following quinidine.[9]

The questions may be asked: Is the effect of quinine only directly on the myocardium and the conduction system, or are the resulting electrocardiographic changes aggravated by anoxemia and the depressive action of quinine on the medullary centers? It would appear to us that anoxemia is not the primary mechanism in the cardiac response since monkeys with a severe malarial infection are anoxic, and their electrocardiographic responses are essentially

the same as those of normal monkeys. The direct cardiac effects of quinine and the relative coronary insufficiency resulting from anemia, hypotension, and respiratory insufficiency, however, may be additive.

A second problem in the mechanism of death in these malaria-infected monkeys is the relationship of anemia to the action of quinine on the respiratory center. The manner of death is the same in monkeys with a severe malarial infection given a small amount of quinine as it is in a normal monkey given twice as much quinine. In man it is well known that the respiratory volume and the cardiac output are increased in cases of severe anemia. This implies increased work and fatigue of both the respiratory center and the heart. Large doses of quinine markedly depress both the heart and respiratory centers, especially in monkeys with a severe anemia. The electrocardiographic changes in dogs with a severe and irreversible anoxia[10] are similar to our findings in monkeys following a lethal dose of quinine. As previously stated, the electrocardiograms were essentially normal in our monkeys with a severe malarial infection, although they were anoxemic. This apparent discrepancy in the electrocardiographic changes in these dogs and monkeys may be explained by the fact that the degree of anoxemia in our malaria-infected monkeys apparently had not reached the irreversible stage, while the dogs studied by Swann[10] were in extremis at the time the tracings were obtained. Experiments are now in progress to study the relation of anemia to the effects produced by quinine.

SUMMARY

1. Monkeys with a severe anemia resulting from *P. knowlesi* infection succumb to a smaller dose of quinine given intravenously than normal animals or those with a less severe anemia.

2. The electrocardiographic changes following equivalent doses of quinine in malaria-infected monkeys follow the same pattern as in normal monkeys, but show evidence of greater cardiodepression.

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A NEW INTRAMUSCULAR PREPARATION OF QUINIDINE (QUINIDINE GLUCONATE)*

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THE object of this communication is to report our experience with the use of an intramuscular preparation of quinidine which we believe has certain advantages over that at present available. The value of quinidine in the treatment of certain cardiac arrhythmias is well recognized. In most of these, the preparation available for oral use, quinidine sulphate, is adequate. However, in certain acute or paroxysmal arrhythmias, the abnormal rate and rhythm may cause cardiac pain, intense palpitation, dyspnea, vomiting, a shocklike state, and even death. In these conditions where quinidine is of recognized efficacy, parenteral administration, because of its more rapid absorption and certainty of effect, has certain advantages over oral administration. In addition, other indications for parenteral administration are vomiting and diarrhea, moderate or severe grades of congestive failure when absorption by mouth is slow and uncertain, and the presence of any condition (postoperative, for example) which might prevent the patient from taking food by mouth.

The intravenous preparations of quinidine which are available are quinidine hydrochloride and quinidine lactate. Our experience with the intravenous preparations is limited to relatively few patients because of their possible harmful effect.

We have had a rather extensive experience with the intramuscular preparations of quinidine over a period of years. We have found the intramuscular method to be a satisfactory and a relatively safe one for administration of this drug. Sagall, Horn, and Riseman[1] have shown that following an intramuscular injection of 5 grains of quinidine hydrochloride, a definite response is observed in fifteen minutes and occasionally in five minutes. The maximum effect of the quinidine injected intramuscularly was obtained in one and one-half hours. As far as we are aware, the only intramuscular preparation at present available is that reported by Sturnick, Riseman, and Sagall,[2] in which quinidine hydrochloride is buffered with urea and antipyrine. We have found this preparation to be quite satisfactory. However, in some of the lots which we have had prepared, the salt has settled out and some of the injections have been painful.

We have recently used quinidine gluconate for intramuscular injection and have found the results obtained to be quite satisfactory. The formula for this preparation is:

$$C_{20}H_{24}N_2O_2.C_5H_6(OH)_5COOH$$

From The Division of Cardiology, Philadelphia General Hospital, and The Robinette Foundation, University of Pennsylvania.

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*The drug was supplied by the Physician's Drug and Supply Company, Philadelphia, Pa.

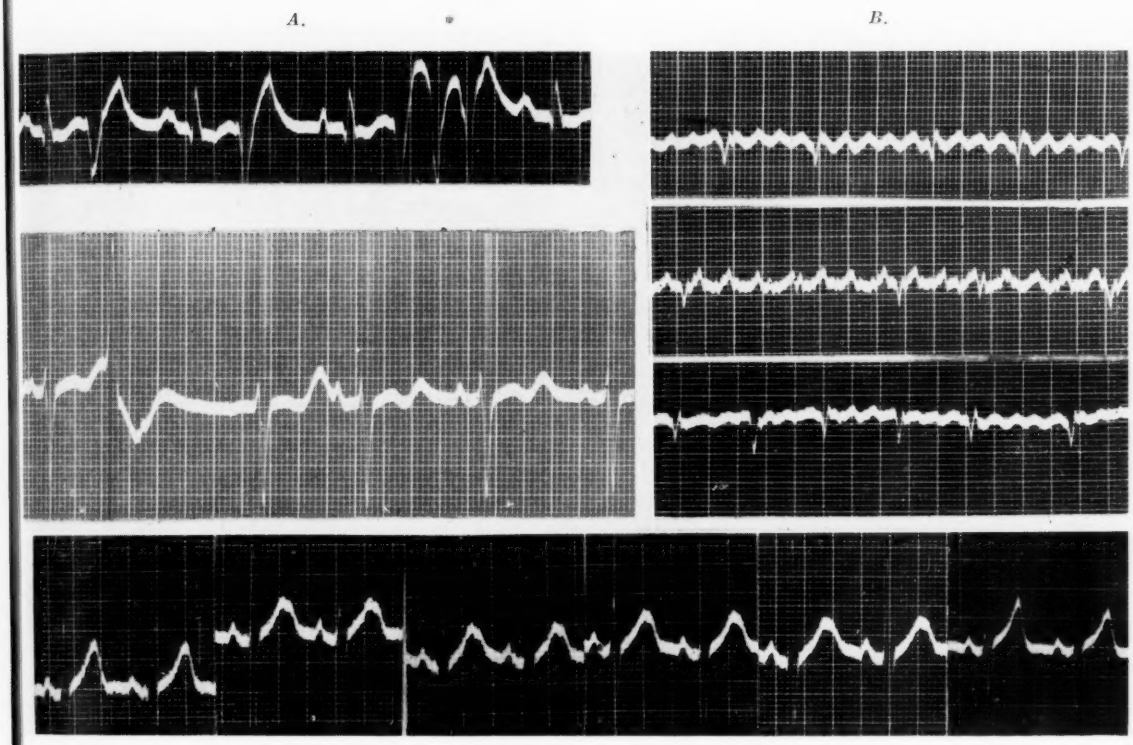

Fig. 1.—*A*, G. W., 62-year-old man. Diagnosis: arteriosclerotic heart disease, congestive heart failure, and diabetes. (Lead CR_3.) Upper strip shows evidence of digitalis intoxication. Many extrasystoles are noted, three of them occurring in succession. Lower strip, four hours after 7½ grains of quinidine gluconate have been given intramuscularly, shows only occasional ventricular extrasystoles and a return to a predominantly normal rhythm.

B, M. D., 48-year-old woman. Diagnosis: rheumatic heart disease, mitral stenosis, cardiac enlargement, and auricular fibrillation. (Lead CR_3.) Upper control strip shows auricular fibrillation with a ventricular rate of about four hundred per minute. Middle strip, one hour after 5 grains of quinidine gluconate had been given intramuscularly, shows that the auricular rate has slowed to three hundred per minute with the production of auricular flutter. The slowing of the auricular rate in auricular fibrillation is a characteristic quinidine effect. Lower strip, eleven hours after quinidine gluconate had been given, shows a return of the auricular fibrillation.

C, A. D., 48-year-old man. Heart normal. Lead CR_4 was taken. Seven and one-half grains of quinidine gluconate were administered intramuscularly and electrocardiograms repeated at fifteen-minute intervals for three hours and at one-hour intervals thereafter. The segments of the record are from right to left: control, fifteen minutes, thirty minutes, two hours, two and three quarter hours, and twenty-four hours after quinidine was administered. Note the initial effect of quinidine action observed in the tracing taken fifteen minutes after injection. Note the notched T wave and the slight lengthening of the QT interval which continues for several hours. The tracing taken twenty-four hours after the injection shows a return to normal with absence of quinidine effects.

The molecular weight is 520.55; it contains anhydrous quinidine 62.3 per cent and gluconic acid 37.7 per cent. It occurs as a white, dextrorotatory powder which is soluble in nine parts of water. The solution is neutral or slightly alkaline in reaction.[3]

This preparation has been used by us in various cardiac states in fifteen patients, some of whom received as many as ten injections of 150 mg. at varying intervals. This preparation appears to have some advantages over that mentioned. The solution is stable; the preparation is relatively simple in type; it results in no irritation at the site of the injection; and the administration of

other compounds (for example, antipyrin, the action of which may be unwanted and undesirable) is not necessary. Characteristic quinidine effects were obtained clinically. These consisted of abolition of ventricular extrasystoles, slowing of the auricular rate in auricular fibrillation, and the production of characteristic changes in the T waves and Q-T intervals in patients with normal sinus rhythm. We have obtained observable effects within fifteen minutes following the injection of a 5 to 7½ grain dose of the drug. (Fig. 1, *A*, *B*, and *C*.)

SUMMARY

Our experience with an intramuscular preparation of quinidine (quinidine gluconate) has been reported. The solution is stable, is simple in type, and results in no irritation at the site of the injection. A characteristic quinidine effect is observed in a relatively short period of time after injection, as early as fifteen minutes after a dose of 5 to 7½ grains. Absorption by the intramuscular route is certain, uniform, and relatively safe. This route of administration is suggested for those patients in whom a rapid effect is required or in whom its administration by the oral route is not considered feasible.

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STUDY OF COMPLETE PARENTERAL ALIMENTATION ON DOGS

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THE present study was undertaken in an effort to improve the nutritional status of patients who cannot receive food by mouth but who must be fed parenterally. Such patients require a nutrient supply not only adequate in caloric content but also one which contains sufficient water, minerals, vitamins, carbohydrate, protein, and fat. Each of these six nutritional elements requires the presence of the other five before it can be properly utilized.

Intravenous feeding of glucose, saline, some of the vitamins, and amino acids or protein hydrolysate has been common practice for some time but has not proved to be completely satisfactory because of the lack of fat. It has been difficult to obtain a fat emulsion suitable for intravenous use, and the literature contains only five reports[1-5] of attempts to use parenteral alimentation using all three of the energy-producing foodstuffs. In these reports either the diet was incomplete or the period of feeding too short for adequate evaluation of results.

Recent work[6] has led to the development of a stable, fine fat emulsion which is believed to be suitable for intravenous use and should overcome the difficulties described. A complete intravenous diet was prepared using this emulsion and a protein hydrolysate. It was administered to dogs over a period of ten weeks during which time they received food exclusively by vein.[7] Results were generally very satisfactory, but the animals showed intermittent hematuria and a mild anemia.

It was felt that the hematuria might have been due to the mechanical difficulties in voiding. The present study was carried out in an attempt to avoid the previous difficulties and demonstrate the possibility of maintaining animals completely healthy by complete parenteral alimentation.

EXPERIMENTAL PROCEDURES

The fat emulsion used in this experiment was prepared as described in the previous communication.[6] However, its composition was altered slightly. It is shown in Table I.

Five healthy adult dogs were used in this experiment. The animals were confined in individual metabolism cages which permitted a quantitative collection of urine. In the control period they were fed a complete basal diet (Table II) which furnished 80 calories per kilogram of body weight per day of which 50 per cent came from carbohydrate, 16 per cent from protein, and 34 per cent from fat. Immediately following this period, four animals (Dogs B, R, C, and M) were given a diet containing the same amount of carbohydrate, protein, and fat, but it was infused exclusively by vein. Carbohydrate was furnished in the form of glucose, protein in the form of Amigen,* and fat as 10 per cent olive oil emulsion.

From the Department of Physiology, Vanderbilt University Medical School.

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*Amigen, a protein hydrolysate, was furnished through the courtesy of Dr. Warren M. Cox, Jr., Mead Johnson & Company, Evansville, Ind.

TABLE I. COMPOSITION OF FAT EMULSION

CONSTITUENT	AMOUNT
Pure olive oil*	10.0 Gm. %
Span 20	0.5 Gm. %
Asolectin	0.4 Gm. %
Sodium cholate	0.1 Gm. %
Glucose	5.0 Gm. %
Vitamin A†	770 units
Vitamin D†	110 units
Alpha-tocopherol acetate	3.5 mg.
Distilled water	85 ml.

*Pure virgin olive oil.

†Supplied as Oleum Percomorphum, a product of Mead Johnson & Company, Evansville, Ind.

TABLE II. COMPLETE BASAL DIET

CONTENT	AMOUNT/KG. PER DAY	CAL./KG. PER DAY	% CALORIES
Sucrose	10.00 Gm.	40.0	50.0
Casein	3.30 Gm.	13.2	16.0
Yeast	0.90 Gm.	—	—
Celluration	0.90 Gm.	—	—
Salt mixture*	0.42 Gm.	—	—
Lard	3.00 Gm.	27.0	34.0
Vitamin A	250 U.S.P. units	—	—
Vitamin D	36 U.S.P. units	—	—
Total	18.52 Gm.	80.2	

*Wesson's salt mixture.[8]

Minerals and vitamins other than A, D, and E were supplied also. The composition and amount of mineral and vitamin mixtures infused into the animals in this study are shown in Tables III and IV. Water was allowed by mouth ad libitum and liver extract* and folic acid† were given intramuscularly every week. The fifth dog (Dog Y) received the same amount of carbohydrate, protein, minerals, and vitamins but without the fat. The caloric intake of Dog Y during the parenteral feeding period was inadequate (53 calories per kilogram per day).

TABLE III. SALT MIXTURE

CONSTITUENT	GM./KG. PER DAY
Sodium chloride	0.400
Potassium iodide	0.001
Calcium gluconate	0.250
Magnesium chloride	0.025
Potassium chloride	0.02
Ferric chloride	0.003
Copper chloride	0.0007
Potassium acid phosphate	0.048
Sodium phosphate, dibasic	0.150

TABLE IV. VITAMIN MIXTURE

CONSTITUENT	MG./KG. PER DAY
Thiamine HCl	0.20
Riboflavin	0.10
Pyridoxine HCl	0.06
Calcium pantothenate	0.20
Choline chloride	10.00
Inositol	2.00
Nicotinamide	2.00
Hykinone	0.10

*Liver Injection U. S. P., obtained from Division of Wilson and Company, Inc., Chicago, Ill. One milliliter (10 U. S. P. units) was given weekly.

†Folvite, a product of Lederle Laboratories Division, American Cyanamid Company, New York, N. Y. Two milliliters (15 mg. per milliliter) were given every week.

The effects of complete parenteral alimentation on hematologic changes, body weight, nitrogen balance, "water balance," liver function, total plasma protein, plasma nonprotein nitrogen, and urinary sugar and albumin were followed. The same studies also were carried out for three or four weeks prior to the injections.

Methods of chemical determinations and hematologic study performed in this experiment are as follows. The nitrogen content of urine and diet was determined by the semimicro-Kjeldahl method. The nitrogen content of feces was calculated as 12 per cent of the urinary nitrogen excretion based on the previous experimental results.[9] The nitrogen balance in the three-day period was determined. The "water balance" was simply measured by the differences between the volume of fluid intake (water by mouth or water by mouth plus infused fluid) and the volume of urinary output. It also was conducted over a three-day period. A blood sample for hematologic study was drawn on Monday morning before the infusion started in all instances (approximately thirty to forty hours after the previous injection of the six-day-a-week schedule). Blood hemoglobin was determined by Sahli's method. Red and white blood cells were counted in the accepted manner and the hematocrit was obtained by centrifuging the oxalated blood in a Wintrobe tube for half an hour at 2,500 revolutions per minute. Body weight was obtained just prior to the blood sampling for hematologic study every Monday.

The methods used for determining total plasma protein, plasma nonprotein nitrogen, rose bengal dye clearance test, serum phosphatase, and urinary sugar and albumin were the same as described in the previous communication.[6]

A solution containing 10 per cent Amigen, 15 per cent glucose, and the required minerals and vitamins was mixed and infused as one solution. The olive oil emulsion was infused separately and in all instances was infused before the other mixture. Daily infusion was completed in approximately nine to ten hours (three to four hours for fat emulsion and six to seven hours for Amigen, glucose, vitamins, and minerals). The infusion rate of glucose, protein, and fat was approximately 1.4, 0.55, and 0.86 Gm. per kilogram of body weight per hour respectively.

During infusion, the animals remained in the individual cages. The use of the swivel of Jacobs[10] with modification permitted the animal complete freedom of movement during the infusion. Infusions were carried out by introducing polyethylene tubing* (0.025 by 0.004 in.) into the external jugular vein. The tubing was left in the vein for two to five weeks. The dogs were infused for four weeks and then sacrificed for histologic study.

RESULTS

General Appearance.—All animals (Dogs B, R, C, and M) which received complete parenteral alimentation including the fat remained healthy, lively, and in good spirits throughout. Their hair was smooth and shining. However, Dog Y, which received no fat, appeared very emaciated and apathetic. Its hair was very coarse and dry, and epilation was starting to occur on the hind legs. The conditions are shown in Figs. 1 and 2.

Hematologic Studies.—In Table V are shown the hematologic values of the five animals (Dogs B, R, C, M, and Y) determined during the control period. Table VI presents the average deviation of the weekly hematologic findings of Dogs B, R, C, and M during the experimental period from those determined during the period of control. The deviations of the weekly findings during the experimental period of Dog Y are also shown in Table VI.

*Polyethylene tubing was obtained from the Irvington Varnish and Insulator Company, Irvington, N. J.

The differences in white cell count and hemoglobin determination in Dogs B, R, C, and M during the entire four-week period of complete parenteral alimentation with the fat were not significant. However, these dogs showed a slight fall in red cell count after the fourth week and a slight fall in hemato-

Fig. 1.—A photograph of Dog B which was taken at the end of the four-week period of complete parenteral alimentation including the fat.

TABLE V.* HEMATOLOGIC VALUES DURING THE CONTROL PERIOD†

DOG	BLOOD PICTURE			
	R.B.C. (MILLION PER CU. MM.)	W.B.C. (PER CU. MM.)	HB. (GM. %)	HEMATOCRIT (%)
B	6.03	17,350	14.2	44.5
R	6.96	14,517	13.6	44.5
C	6.04	15,400	13.9	38.0
M	5.27	9,988	13.7	37.0
Y	4.58	10,180	12.3	34.0

*Acknowledgment is made to Dr. Margaret P. Martin, Vanderbilt University Medical School, for statistical analysis of the data presented in Tables V, VI, and VII.

†For red blood cell count and white blood cell count the average of three or four weekly observations for each dog was computed. For hemoglobin and hematocrit the last value of the three or four weekly observations for each dog was used.

crit after the third week of infusion, which is statistically significant. Dog Y, the animal which received parenteral alimentation without fat, also showed a slight reduction in hematocrit after the third week and a marked reduction after the fourth week of infusion.

Fig. 2.—A photograph of Dog Y which was taken at the end of the four-week period of parenteral alimentation without the fat.

TABLE VI. DEVIATION FROM THE VALUES DETERMINED DURING THE CONTROL PERIOD

WEEK (EXPERIMENTAL)	BLOOD PICTURE			
	R.B.C. (MILLION PER CU. MM.)	W.B.C. (PER CU. MM.)	HB. (GM. %)	HEMATOCRIT (%)
Average Deviations for Dogs B, R, C, and M				
1	+0.04	+ 924	−0.25	+0.4
2	−0.32	+1,336	−0.35	−2.5
3	−0.50	+2,599	−0.65	−4.5
4	−0.74	+2,512	−0.60	−4.5
Sampling variation of each of these averages $\pm 2\sigma\eta$	±0.54	±3,282	±1.24	±2.56
Deviations for Dog Y				
1	−0.36	−1,330	−1.00	0
2	+0.37	−1,330	−0.2	0
3	+0.27	+3,770	0	−2.0
4	+0.11	+10,180	−1.00	−3.0

Body Weight and Nitrogen Balance.—All four animals (Dogs B, R, C, and M) receiving complete parenteral alimentation maintained or slightly increased body weight during the period of infusion. However, Dog Y which

received the same nutrient medium but without the fat lost 14 per cent of its initial weight. The nitrogen balance was slightly variable during the period of infusion. Some of the animals showed negative balance during the first

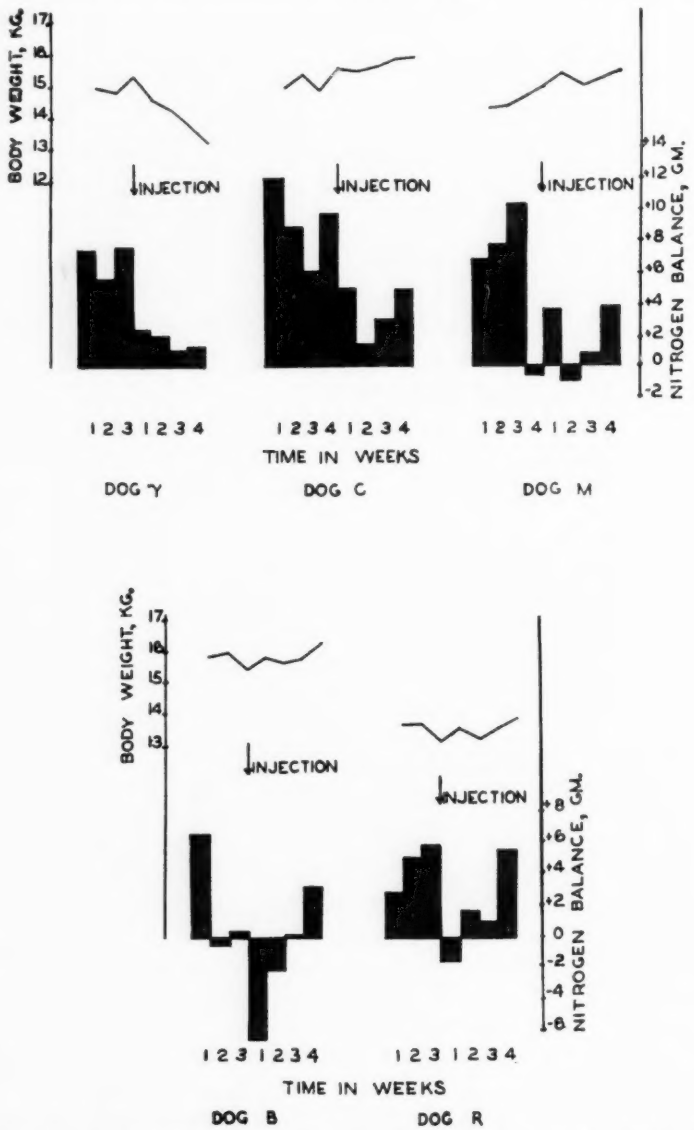

Fig. 3.—Showing the nitrogen balance and change in body weight during the complete parenteral alimentation period (Dog Y received no fat in the nutrient medium) similar to those during the control period.

(Dog R) or second (Dog M) or both first and second (Dog B) weeks of infusion. However, negative balance also was observed on Dogs B and M during the second and fourth week respectively of the control period. The nitrogen balance of Dog Y during infusion was less positive than that during the control period. The results are shown in Fig. 3.

"*Water Balance.*"—During the control period the average "water balance" of the three-day interval scheme for Dogs B, R, C, and M was +818 ml. (ranging from 634 to 985 ml.). The same animals averaged +1,447 ml. (ranging from 969 to 1,938 ml.) for the experimental period. The same change took place in Dog Y. The significance of these observations is doubtful since no account was taken of the insensible loss. There was probably no true water retention since there was no evidence of edema. The results of this study are presented in Table VII.

TABLE VII. SUMMARY OF THE "WATER BALANCE"

DOG	PERIOD	NUMBER OF WEEKLY OBSERVATIONS	INTAKE (ML.) 3-DAY			OUTPUT OF URINE (ML.) 3-DAY (AVERAGE)	BALANCE (ML.) 3-DAY) (AVERAGE)
			WATER BY MOUTH (AVERAGE)	FLUID INJECTED (AVERAGE)	TOTAL (AVERAGE)		
B	Control	1	2,260	——	2,260	1,420	+ 840
R	Control	1	1,890	——	1,890	1,030	+ 860
C	Control	4	1,708	——	1,708	723	+ 985
M	Control	4	1,643	——	1,643	1,009	+ 634
Average for 4 dogs		10	1,755	——	1,755	938	+ 818
B	Exper.	4	1,454	3,990	5,444	4,475	+ 969
R	Exper.	4	1,860	3,420	5,280	4,235	+1,045
C	Exper.	4	2,780	3,750	6,530	4,695	+1,835
M	Exper.	4	2,285	3,660	5,945	4,008	+1,938
Average for 4 dogs		16	2,095	3,705	5,800	4,353	+1,447
Y	Control	3	2,070	——	2,070	1,240	+ 830
Y	Exper.	4	3,453	2,550	6,003	4,690	+1,308

Blood Chemistry and Liver Function.—There was no change in total plasma protein concentration. The plasma nonprotein nitrogen remained within the normal range throughout the infusion period except in the case of Dog M which showed a slight increase. However, there is no basis to assume that such change was due to renal damage incident to those infusions since the findings observed during the control period were high. The rose bengal clearance test was reduced in three of the four dogs (Dogs B, C, and M) receiving complete parenteral alimentation. Serum phosphatase was somewhat elevated in all the animals of this group. The animal (Dog Y) which received no fat demonstrated the same changes. However, the serum phosphatase determinations made just before the animals were sacrificed showed lowered or normal levels in all five dogs. These results are shown in Table VIII. Neither sugar nor albumin was detected in the urine throughout.

*Histologic Study.**—Gross examination revealed no abnormalities at the end of the four-week infusion period in both groups of animals (with or without the fat). Microscopically, Dogs B, R, C, and M, the animals receiving complete parenteral alimentation, showed a few stainable fat granules in some of the Kupffer cells. However, the parenchymal cells were fat-free and normal. There was no fat observed in the liver of Dog Y. In the kidney sections of all five animals including the dog (Y) receiving no fat, there was positive fat stain in the cytoplasm of some of the distal convoluted tubules. The

*Acknowledgment is made to Dr. James R. Dawson, Jr., Professor of Pathology, Vanderbilt University Medical School, for interpretation of the histologic sections.

glomeruli appeared normal and no fat was demonstrated. The sections of the lung of Dogs R, M, and Y showed normal structure and were fat free. There were three or four fat emboli observed in the lung section of Dog C; otherwise it was essentially normal. There was a very small ulcer which was in the acute stage seen in the section of the duodenum of Dog M, but no similar finding was seen in the sections of the other animals. Other sections revealed normal heart, pancreas, stomach, spleen, bone marrow, and adrenal and thyroid glands in all five experimental animals.

TABLE VIII. EFFECT ON LIVER FUNCTION, TOTAL PLASMA, PROTEINS, AND PLASMA NONPROTEIN NITROGEN

DOG	PERIOD	WEEK	TOTAL PLASMA PROTEINS (GM. %)	PLASMA N.P.N. (MG. %)	ROSE BENGAL CLEARANCE (%)	SERUM PHOSPHATASE* (ALKALINE)
B	Control	1	–	–	–	–
		2	7.27	24.5	104.0	9.45
		3	7.15	23.6	94.0	8.73
	Exper.	1	–	–	–	–
		2	7.06	24.6	86.0	17.3
		3	–	–	–	–
		4	6.6	29.3	76.0	12.0; 10.9†
R	Control	1	–	–	–	–
		2	6.83	21.1	123.0	5.58
		3	6.55	21.8	110.0	5.83
	Exper.	1	–	–	–	–
		2	6.96	34.3	102.0	17.3
		3	–	–	–	–
		4	6.6	22.1	102.0	8.4; 6.8†
C	Control	1	7.6	20.0	93.0	4.9
		2	7.2	21.7	–	6.3
		3	7.3	21.3	83.0	7.7
	Exper.	1	–	–	–	–
		2	5.9	33.5	73.5	10.1
		3	–	–	–	–
		4	6.1	27.2	92.5	6.4; 6.9†
M	Control	1	6.8	56.4	97.0	4.3
		2	6.7	56.9	97.2	5.3
		3	7.6	64.7	90.0	5.6
	Exper.	1	–	–	–	–
		2	5.7	72.1	65.3	10.6
		3	–	–	–	–
		4	6.0	72.5	74.0	9.5; 8.2†
Y	Control	1	5.9	40.7	86.0	2.7
		2	6.2	42.0	62.2	3.6
		3	6.7	41.9	77.0	4.2
	Exper.	1	–	–	–	–
		2	6.3	32.9	69.7	4.6
		3	–	–	–	–
		4	7.0	30.3	44.0	10.3; 5.0†

*Bodansky units per 100 milliliters.
†Just before animals were sacrificed.

DISCUSSION

The animals used in the previous experiment[7] were injected while fastened on their sides on a canvas frame. It is obvious that the urinary bladder became very much distended since the animals were not accustomed to void in

such a position. Hematuria resulted simply by rupture of the blood capillaries of the urinary bladder due to mechanical stretching. This was proved to be true by autopsy findings. By using the swivel device in this study, the animals were permitted to move at will. There was not the slightest difficulty encountered in urination or defecation and hematuria has never been observed.

Although the results showed a statistically significant fall of red cell count and hematocrit, it is reasonable to believe that the fat emulsion infused was not responsible for the slight change. In the first place, the observed average decrease for red cell count of Dogs B, R, C, and M was only −0.74 after the fourth week of complete parenteral alimentation with the fat. Second, Dog Y, the animal which received no fat, also demonstrated the reduction of hematocrit (Tables V and VI). Such small changes would not be surprising in view of the change of diet and degree of hydration. Finally, histologic examinations showed essentially normal bone marrow in all the experimental animals which would further support the belief.

The reduction of rose bengal clearance and elevation of serum phosphatase observed during complete parenteral alimentation indicate the impairment of liver function. However, the changes were demonstrated in all five dogs including the one which received no fat. The serum phosphatase determinations made just before the animals were sacrificed gave values which were at the control level (Table VIII). In view of this fact it seems reasonable to state that the signs of liver impairment were not caused by the intravenous administration of fat but rather were due to the complete parenteral alimenation in general since Dog Y, which received no fat, demonstrated the same result. It is quite likely that during the infusion period the liver was under a heavy burden and had to work extremely hard in order to carry out the necessary processes for the utilization of the nutrients intravenously introduced. Furthermore, the histologic examination of the liver showed normal parenchymal cells which again rules out the possibility of severe liver damage. The presence of a few fat granules in some of the Kupffer cells of the liver is probably as one might expect. However, it might be suggested that in clinical use if a patient's liver function is severely impaired, parenteral alimentation of any kind be given only after careful consideration.

The stainable fat found in the renal tubules is probably not the result of intravenous administration of fat. There was more stainable fat demonstrated in the tubules of Dog Y, the animal receiving no fat, than in those of the fat-infused animals. Albumin was never detected in the urine, and surprisingly enough glycosuria was never observed during the complete parenteral alimentation although the infusion rate of glucose was approximately 1.4 Gm. per kilogram of body weight per hour. Woodyatt and associates[11] reported that 0.85 Gm. per kilogram per hour (in 10 to 50 per cent solution) was the maximum speed of intravenous infusion of glucose without production of glycosuria. Lockhart and Elman[12] recently found that in human subjects 0.5 Gm. per kilogram per hour was the maximum speed of infusion with-

out production of glycosuria. This may be interpreted in part, at least, as being due to the following factors. (1) The addition of protein hydrolysate to the glucose solution increases the rate of utilization of glucose by the tissues as suggested by Lockhart and Elman.[12] (2) The addition of vitamin and mineral mixtures as carried out in the present study may further enhance the glucose-protein hydrolysate utilization. (3) The rate of glucose utilization may be faster in the dog than in man. Nevertheless, the absence of albuminuria and glycosuria demonstrates that there was no impairment of renal function which can be attributed to the complete parenteral alimentation.

The increase in urinary volume output during parenteral alimentation was simply due to the intravenous administration of a large quantity of fluid. The water intake by mouth of Dogs B, R, C, and M varied very little, with an average of 1,755 ml. for the three-day interval scheme during the control period. Even during the experimental period, oral water intake of Dogs B and R was in this range. However, water intake by mouth of Dogs C and M was increased during the experimental period. The average water intake by mouth of Dog Y was 2,070 ml. during the control period as compared with the average of 3,453 ml. during the experimental period. The increase of water intake by mouth of Dogs C, M, and Y was probably due to the elevation of room temperature during the first part of the winter when the rooms were first heated. The water loss by channels other than urinary output, e.g., panting, was increased, leading to an increase of "water balance." However, it is not a true retention. The average "water balance" of Dogs B, R, C, and M during the control period was +818 milliliters. The average amounted to +1,447 ml. during the experimental period for the same group of animals. The average increase of "water balance" for the three-day interval scheme was 629 ml. which is only 209 ml. per day. The average "water balance" of Dog Y, the animal receiving no fat, was also increased during the experimental period (428 ml. for three-day period) (Table VII). It is our belief that there was no disturbance in body fluid of different compartments during parenteral alimentation (with or without fat) in the present study.

The experiment of omitting fat from the diet for complete parenteral alimentation of Dog Y was planned in an attempt to obtain further evidence for the utilization of the intravenously administered fat. The animal (Dog Y) received inadequate caloric intake during the infusion period. It is assumed that if the infused fat is not utilized in the body then the results demonstrated by the two groups of animals (with or without the fat) should be similar. It is true that part of the intravenously administered protein hydrolysate is lost in the urine. Unfortunately, determination of amino nitrogen in the urine was not carried out in this study. However, it was probably not more than 10 per cent, since most investigators have shown it will not exceed this if the rate of infusion is not too rapid. The loss of amino nitrogen by means of the kidney might account for the difficulty in establishing nitrogen balance during parenteral alimentation. Nevertheless, the two groups of animals (with or without the fat) should appear similar as far as the nitrogen

loss is concerned. In view of these findings it is logical to believe that the injected fat is utilized in the body for energy. Dog Y lost 14 per cent of its initial body weight and appeared apathetic and emaciated, while the other animals which received the fat were healthy, lively and in good spirits and maintained or increased their body weights (Fig. 3). The gain in body weight of the animals receiving fat was not due to the deposition of the injected fat as adipose tissue as has been demonstrated by McKibbin and associates.[13] Moreover, the dry, coarse hair and the epilation of the hind legs of Dog Y were probably due to a deficiency of the essential fatty acids as demonstrated by Evans and Burr,[14, 15, 16] Burr and co-workers,[17, 18, 19] and by Evans and Lepkovsky[20, 21] on rats, and by Hansen and Wiese[22] on dogs, since low caloric intake (53 calories per kilogram per day) is probably not the only factor responsible for the general picture. Mann and associates[23] showed additional evidence of utilization of the intravenously injected fat in growing puppies. More recently Geyer and co-workers[24] and Lerner and co-workers[25] were able to demonstrate the presence of C^{14} from the collected expired CO_2 from rats after intravenous injection of C^{14} labeled trilaurin and tripalmitin fat emulsions. However, further investigation should be carried out in this regard with adequate and inadequate caloric intake when fat is omitted in the parenteral basal ration to show the dispensableness of the fat.

The cause of the small ulcer observed in the duodenum section of Dog M is obscure. At any rate, there is no reason to suspect that it is related to complete parenteral alimentation. The presence of three or four fat emboli in the lung section of Dog C is certainly not significant insofar as the clinical picture is concerned, since the animal was normal in all other aspects. The failure to show any pathologic changes in other histologic sections indicates that this emulsion could be used clinically with considerable confidence.

SUMMARY AND CONCLUSIONS

Dogs have been maintained for as long as four weeks on a diet administered exclusively by vein. The diet furnished proportional amounts of carbohydrate, protein, fat, minerals, and vitamins. Thirty-four per cent of the caloric content of the diet was furnished by a special fat emulsion. The dogs remained healthy, lively, and in good spirits throughout, and most of them gained weight. Extensive physiologic tests during the course of the experiments, and a complete histologic study following sacrifice, failed to reveal any significant abnormalities attributable to the procedure.

One dog was given the same treatment as the others except that the fat was omitted from the diet. It lost 14 per cent of its initial weight, appeared apathetic and emaciated, and developed lesions suggestive of a fatty acid deficiency. The behavior of this dog gives considerable confidence to the belief that the injected fat in the other dogs was fully utilized, and that its presence in the medium enhances the value.

It is concluded that this emulsion could be used clinically with considerable confidence, and that it would be very beneficial in cases where complete parenteral alimentation is necessary.

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THE NUTRITIVE VALUE OF INTRAVENOUSLY ADMINISTERED HYDROLYZED HUMAN SERUM ALBUMIN IN MAN

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HUMAN serum albumin serves as a protein nutrient for human subjects when administered in the intact form by mouth or by vein.[1, 2, 3] The present study was undertaken to determine its nutrient value when administered intravenously in the hydrolyzed form as amino acids. The results indicate that the parenterally administered hydrolyzed albumin, supplemented with *l*-tryptophane, was nutritionally adequate for a slightly undernourished subject and was metabolized and excreted in a manner similar to that of other hydrolysates.

MATERIALS AND METHODS

The subject selected for the study was a 43-year-old man who was admitted to the hospital ten days previously for acute alcoholism. His general health in the past had been excellent, although during his intermittent alcoholic sprees he drank heavily and ate poorly and had recently lost 10 pounds in weight. The physical examination and laboratory findings, including tests of liver function, were entirely within the limits of normal. The subject was asymptomatic throughout the twenty-third day period of study while hospitalized on the Thorndike metabolic ward.

A 50 Gm. protein diet was used in the first period and supplied 3,000 calories daily. It contained the foods ordinarily eaten in a well-balanced, nutritious diet. The nitrogen content was calculated from standard food tables. In the second period a protein-free diet was used which supplied 3,500 calories but only 0.4 Gm. of nitrogen daily as determined by macro-Kjeldahl analysis; the diet has been described previously.[2]

The hydrolyzed albumin solution was prepared by the complete acid hydrolysis of human serum albumin and was supplemented with *l*-tryptophane.† The composition of the solution and the quantity of amino acids infused are given in Table I. Previously reported values for the amino acid content of casein[4] and of human serum albumin[5, 6] are listed for comparison. One liter of the 7 per cent solution was administered intravenously once daily, together with 100 c.c. of 50 per cent dextrose. The injection was made one hour after a 1,000 calorie breakfast in order to achieve maximum utilization of the infused amino acids by the simultaneous provision of adequate carbohydrate and protein.[7]

The daily urine and pooled stool nitrogen analyses were determinad by the standard micro- or macro-Kjeldahl methods. The urinary alpha amino nitrogen was determined by the gasometric ninhydrin method described by Van Slyke, MacFadyen, and Hamilton.[8] The amino acid analyses of the urine and of the albumin hydrolysate solution were by the microbiologic method of Stokes and co-workers.[4]

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†The human serum albumin was processed and supplied by the Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa., from blood collected by the American National Red Cross from voluntary donors. The hydrolysate was specially prepared for this investigation by acid hydrolysis of the albumin which had been rejected for clinical use because of pyrogen content. The hydrolysis, preparation, and analysis of the pyrogen-free 7 per cent solution were done under the direction of Dr. E. E. Howe, Merck & Co., Inc., Rahway, N. J. The authors express their thanks to the individuals and organizations concerned.

TABLE I. AMINO ACID COMPOSITION OF THE HYDROLYZED HUMAN SERUM ALBUMIN SOLUTION

AMINO ACID	HYDROLYZED HUMAN SERUM ALBUMIN*		HUMAN SERUM ALBUMIN†	CASEIN‡
	GM./INJECTION	GM./100 GM.	(GM./100 GM.)	(GM./100 GM.)
Arginine	4.5	6.5	6.2	3.9
Histidine	2.4	3.5	3.5	2.8
Isoleucine	1.1	1.6	1.7	5.6
Leucine	8.9	12.8	11.9	9.9
Lysine	7.3	10.5	5.8	7.7
Methionine	0.9	1.3	1.3	2.6
Phenylalanine	5.4	7.8	—	5.9
Threonine	4.0	5.8	5.0	4.2
Tryptophane	1.2	1.7 (Added)	0.19	1.1
Valine	4.7	6.8	—	6.7

*Determinations by microbiologic assay.[4] Prepared for intravenous administration as a 7 per cent solution, and each 1,000 c.c. infusion supplies (by analysis) 69 Gm. of amino acids, 11.1 Gm. of total nitrogen, and 8.7 Gm. of alpha amino nitrogen.

†Values of Brand, Kassell, and Saidel,[5] except isoleucine which is the value of Hegsted, Hay, and Stare.[6]

‡Microbiologic values of Stokes and co-workers.[4]

RESULTS

The solution of hydrolyzed human serum albumin was tolerated well clinically when administered intravenously over a period of approximately two and one-half hours. An attempt to administer the solution more rapidly was followed, on two occasions, by flushing and warmth of the face and upper neck, together with mild nausea. Thrombosis of the infusion vein did not occur.

The nitrogen balance data for the subject are summarized in Table II. Nitrogen equilibrium was maintained during an initial five-day control period while the subject received the basal 50 Gm. protein diet. Supplementation of this diet by the addition of 70 Gm. of hydrolyzed human serum albumin (1,000 c.c. of the 7 per cent solution) intravenously daily for five days resulted in a

TABLE II. NITROGEN BALANCE DATA FOR SUBJECT GIVEN HYDROLYZED HUMAN SERUM ALBUMIN INTRAVENOUSLY

PERIOD OF STUDY (DAYS) AND CONDITION OF STUDY	NITROGEN INTAKE (GM./DAY)		NITROGEN OUTPUT (GM./DAY)		NITROGEN BALANCE (GM./DAY)
	ORAL	I. V.	URINE	STOOL	
1-6 Fore-control	8.1	0.0	6.7	1.2	+0.2
6-11 Hyd. albumin, I.V.	8.1	11.1	11.8	1.2	+6.2
11-17 After-control	8.1	0.0	6.4	1.2	+0.5
17-24 Hyd. albumin, I.V.	0.4	11.1	10.2	0.4	+0.9

sustained positive nitrogen balance averaging +6.2 Gm. daily. The nitrogen balance during a six-day after-control period was comparable with that observed initially, and equilibrium again was attained by the 50 Gm. of dietary protein. During the final seven days of study, the subject received the diet adequate in calories but essentially devoid of protein and 1 liter of the 7 per cent solution of hydrolyzed albumin (70 Gm. of amino acids) intravenously daily as the sole source of nitrogen. Nitrogen equilibrium and extreme constancy of the urinary

nitrogen excretion were observed. The nitrogn balance averaged +0.9 Gm. daily. The subject's body weight remained constant throughout the twenty-three day study period.

The excretion of alpha amino nitrogen and of the ten "essential" amino acids in the urine during each of these periods of study is tabulated in Table III. While receiving 50 Gm. of dietary protein, the subject excreted comparable quantities of amino acids during both the fore- and after-control periods. The urinary excretion of amino acids was considerably increased when hydrolyzed albumin (70 Gm. of amino acids) was administered intravenously, whether as a supplement to the 50 Gm. protein diet or as the sole source of nitrogen. While receiving the parenterally injected hydrolyzed albumin as supplemental protein, the subject showed a loss by excretion in the urine of from 0.2 to 14.2 per cent of the individual "essential" amino acids infused, of 2.7 per cent of the total of the ten "essential" amino acids injected, and of 11.9 per cent of the alpha amino nitrogen administered. These losses were somewhat less when the hydrolyzed albumin was administered intravenously as the sole source of nitrogen.* In this instance, the maximum loss of any individual "essential" amino acid infused was 8.4 per cent and averaged

TABLE III. EXCRETION OF AMINO ACIDS IN THE URINE FOLLOWING INFUSIONS IN HYDROLYZED HUMAN SERUM ALBUMIN

	CONSTANT 50 GM. PROTEIN DIET				PROTEIN-FREE DIET			
		PLUS 70 GM. HYD. ALBUMIN, I. V.				PLUS 70 GM. HYD. ALBUMIN, I. V.		
AMINO ACID	CONTROL (MG./DAY)	MG./DAY	EXCESS (MG./DAY*)	PER CENT EXCRETED†	CONTROL (MG./DAY)	MG./DAY	EXCESS (MG./DAY*)	PER CENT EXCRETED†
Arginine	10.0	39.1	29.1	0.7	10.0	48.4	38.4	0.9
Histidine	123	463	340	14.2	130	331	201	8.4
Isoleucine	5.5	7.7	1.9	0.2	5.5	18.4	12.9	1.2
Leucine	11.5	43.6	32.1	0.4	11.5	53.2	41.7	0.5
Lysine	19.7	397	377	5.2	19.5	140	121	1.7
Methionine	3.7	13.6	9.9	1.1	3.3	10.5	7.2	0.8
Phenylalanine	15.1	58.7	43.6	0.8	15.1	78.3	63.2	1.2
Threonine	14.0	226	212	5.3	13.3	186	173	4.3
Tryptophane	12.3	16.4	4.1	0.3	11.8	10.7	0.0	0.0
Valine	5.4	42.5	37.1	0.8	6.7	51.1	44.4	0.9
Total 10 "essential" amino acids	220	1,307	1,087	2.7	227	928	701	1.7
Alpha amino nitrogen	185	1,221	1,036	11.9	186	678	492	5.7

Milligrams of amino acid or of alpha amino nitrogen in excess of that excreted during the preceding control period (see text footnote).

†Per cent of administered amino acid or of administered alpha amino nitrogen (Table I) excreted in urine.

*The amino acid excretion values listed in the last two columns of Table III are in excess of the amount of alpha amino nitrogen and of individual amino acids excreted during the preceding 50 Gm. protein diet period. Since the subject received the hydrolyzed albumin as the sole source of nitrogen during this final period, these values should be in excess of the amount of amino acids excreted while he was receiving the diet devoid of protein. This was not determined. However, since the minimum "endogenous" amino acid excretion is increased only slightly by the ingestion of protein,[9] the error incurred in the calculation of these values is small, particularly in view of the considerably increased excretion of amino acids following infusions of protein hydrolysates.

1.7 per cent for the total of the ten "essential" amino acids administered, while only 5.7 per cent of the alpha amino nitrogen injected was excreted in the urine. In each instance, however, the subject excreted considerably greater percentages of administered histidine and threonine, and to a lesser extent lysine, than of the other "essential" amino acids in the infusion mixture.

During the periods while hydrolyzed albumin was administered intravenously, the urinary excretion of total nitrogen, of alpha amino nitrogen, and of each of the ten "essential" amino acids promptly increased and remained constant throughout its administration. The day the hydrolysate infusions were discontinued, these values immediately returned to those of the preceding control period. Thus, the data presented in Tables II and III and averaged for each period of study are valid representations of the changes that occurred daily.

DISCUSSION

This study demonstrates that a complete acid hydrolysate of human serum albumin, supplemented with *l*-tryptophane, can maintain weight and nitrogen balance for short periods of time in man when administered intravenously (70 Gm. of amino acids daily) as the sole source of food nitrogen. Furthermore, approximately half of that injected parenterally into the subject as a supplement to a 50 Gm. protein diet was retained and presumably formed extra body tissue. The solution employed, therefore, contained all the essential amino acids required by man for maintenance of nitrogen balance and weight, and for tissue protein repletion, when administered in adequate amounts by vein for short periods of time. In these regards, intravenously administered hydrolyzed human serum albumin was metabolized and utilized in a manner similar to hydrolysates prepared from other protein sources.

The values for the amino acid composition of hydrolyzed human serum albumin reported in this paper and determined by microbiologic assay agree quite closely with those previously reported (Table I).[5, 6] The lysine content was higher than found before, while the phenylalanine and valine contents (7.8 and 6.8 Gm. per 100 Gm. of albumin respectively) have not been published previously.

The low isoleucine and tryptophane content of albumin, particularly when compared with casein (Table I), previously occasioned concern over the protein's nutritional adequacy, especially since albumin was found incapable of supporting growth when fed to young rats until extra quantities of these amino acids were provided.[6] However, no supplementation was required for maintenance of nitrogen balance in the adult dog.[6] Previous studies in this laboratory demonstrated that adequate quantities of orally ingested human serum albumin, not supplemented with isoleucine or tryptophane, maintained nitrogen balance and weight for short periods of time in a normal subject.[2] Furthermore, the provision of additional isoleucine and tryptophane failed to alter nitrogen excretion and balance whether albumin was given by mouth in amounts insufficient to maintain nitrogen equilibrium or by vein in amounts

adequate to achieve positive nitrogen balance.[2] It also has been demonstrated that unsupplemented albumin serves as a protein nutrient when administered in the intact form intravenously.[1, 3] Finally, the nutritional adequacy of albumin administered intravenously in the hydrolyzed form as amino acids, supplemented with *l*-tryptophane, was demonstrated in the present study. Since tryptophane is destroyed upon acid hydrolysis, it was necessary to supplement the solution with this "essential" amino acid, although considerably more was added (1.2 Gm. per liter of 7 per cent solution) than is present in the native protein (0.19 Gm. per 100 Gm. of albumin). Since albumin containing only 0.19 per cent tryptophane is nutritionally adequate when administered in the intact form by mouth or by vein, it would be unlikely that it is inadequate when administered intravenously in the hydrolyzed form as amino acids. Under these conditions, however, somewhat larger quantities of the hydrolysate might have to be provided, since the quantitative requirement of hydrolyzed protein administered intravenously is probably greater than that of an equivalent quantity given orally.[10, 11]

The values for alpha amino nitrogen and for the ten "essential" amino acids excreted in the urine by the subject while receiving the 50 Gm. protein diet (Table II) are in general agreement with those reported previously from this laboratory[9, 12] and by others employing microbiologic methods.[13, 14, 15] These values were considerably increased when hydrolyzed albumin was administered

TABLE IV. COMPARISON OF AMINO ACID EXCRETION FOLLOWING INFUSIONS OF HYDROLYZED ALBUMIN AND HYDROLYZED CASEIN*

	ADMINISTERED				EXCRETED					
	GM./INFUSION		% OF TOTAL		MG./24 HR.		% OF TOTAL		% OF THAT GIVEN	
AMINO ACID	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN
Arginine	4.5	2.5	11.1	8.1	29.1	8.7	2.7	1.2	0.7	0.3
Histidine	2.4	1.6	6.0	5.1	340	206	31.3	28.8	14.2	12.9
Isoleucine	1.1	3.8	2.7	12.6	1.9	9.1	0.2	1.3	0.2	0.2
Leucine	8.9	7.8	22.0	25.7	32.1	45.7	2.9	6.4	0.4	0.6
Lysine	7.3	4.2	18.1	13.9	377	149	34.7	20.8	5.2	3.5
Methionine	0.9	3.2	2.2	10.6	9.9	34.4	0.9	4.8	1.1	1.1
Phenylalanine	5.4	2.7	13.4	8.8	43.6	43.1	4.0	6.0	0.8	1.6
Threonine	4.0	1.1	9.9	3.5	212	155	19.5	21.6	5.3	14.1
Tryptophane	1.2	0.45	3.0	1.5	4.1	22.7	0.4	3.2	0.3	5.7
Valine	4.7	3.1	11.6	10.2	37.1	42.1	3.4	5.9	0.8	1.4
Total	40.4	30.4	100.0	100.0	1,087	716	100.0	100.0	2.7	2.3

*Averaged values following infusions of 500 c.c. of a 10 per cent solution of hydrolyzed casein to two normal subjects in approximately one hour.[12]

intravenously. Since albumin and casein differ in regard to their amino acid composition (Table I), the amino acid excretion following infusions of hydrolyzed albumin was compared with that following infusions of a hydrolysate of casein, previously reported from this laboratory.[12] These data are shown in Table IV. Despite differences in the amino acid composition of these two solutions, quite similar percentages of each administered amino acid were excreted in the urine, and the postinfusion pattern of the "essential" amino

acids was similar whether hydrolyzed albumin or hydrolyzed casein was injected. The individual amino acids, however, were not excreted in the same proportion as administered in either hydrolysate, so that considerably greater percentages of administered histidine and threonine, and to a lesser extent lysine, were excreted than of the other "essential" amino acids. Despite the difficulty in interpreting these observations, as discussed previously,[12] the similarity in the renal excretion of amino acids following infusions of hydrolyzed albumin and of hydrolyzed casein is apparent.

SUMMARY AND CONCLUSIONS

1. One liter of a 7 per cent solution of hydrolyzed human serum albumin, prepared by the complete acid hydrolysis of albumin and supplemented with *l*-tryptophane, was administered intravenously daily to a slightly undernourished subject for five days as a supplement to a 50 Gm. protein diet, and for seven days as the sole source of food nitrogen.

2. Nitrogen equilibrium was maintained when hydrolyzed albumin (70 Gm. of amino acids) was injected intravenously as the sole source of nitrogen, while a sustained positive nitrogen balance was achieved during its administration as supplemental protein. The subject's body weight remained constant throughout the study.

3. The excretion of alpha amino nitrogen and of the ten "essential" amino acids in the urine following the intravenous administration of the hydrolyzed albumin solution was similar to that previously observed following infusions of hydrolyzed casein.

4. It is concluded that parenterally administered hydrolyzed human serum albumin, supplemented with *l*-tryptophane, is nutritionally adequate for man and is metabolized and excreted in a manner similar to that of hydrolysates prepared from other protein sources.

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ABSORPTION OF UNEMULSIFIED AND EMULSIFIED VITAMIN A IN SPRUE

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INTRODUCTION

A CHIEF result of the sprue syndrome is the impaired absorptive power of the small intestine. Among the prominent features of the syndrome are deficiency of hematopoietic essentials, fat-soluble vitamins, minerals, and protein. Diminished absorption of ingested foods, especially fats and fat-soluble substances, is an important cause for the development of the many specific deficiencies. Even though therapy alleviates most of the symptoms, restores the weight, the blood picture, glucose absorption, and gastric acid secretion to reasonably normal levels, there still remains in a number of patients the problem of persistent steatorrhea. Much of the disability in sprue patients can be attributed to steatorrhea. With the steatorrhea, there is impaired absorption of vitamin A, vitamin K, and calcium.

Vitamin A deficiency does not always manifest itself by clinical evidence but may be demonstrated only with biochemical methods. The investigations reported here are concerned only with the aspect of vitamin A deficiency in sprue. A report of numerous other studies conducted on these patients will appear elsewhere.

Because previous investigators[1] had shown that vitamin A absorption was markedly augmented in children with steatorrhea when fed emulsified materials, it was decided to conduct similar studies on the comparable effect of feeding unemulsified and emulsified vitamin A preparations to adult patients with sprue.

MATERIALS AND METHOD OF STUDY

The procedures for doing plasma vitamin A determinations were similar to those previously described.[2, 3] The vitamin A levels herein reported are listed in micrograms. One microgram of vitamin A represents approximately 2.5 I.U. For the unemulsified vitamin A material, oleum percomorphum was used. The vitamin A content of this fish liver oil is rated at 60,000 I.U. per gram and is largely in the form of esters. The emulsified vitamin A material used was a special preparation. The emulsion was composed of 15 per cent vitamin A acetate, 15 per cent Tween 20,* and 70 per cent water. This emulsion contained 100,000 I.U. of vitamin A per gram. Determinations were made on plasma specimens taken fasting and at intervals of three, five, and seven hours after the feeding of a test dose of 200,000 μg of vitamin A material. The peak rise in vitamin A occurred during the three- to five-hour period and fell to lower levels at seven hours. Therefore, only the fasting and the three- and the five-hour levels are included in this report to show the maximum rise. The test dose of vitamin A material used in all these experiments was given to the patients after an overnight fasting period. Milk was used as a vehicle for the materials followed by feeding a normal breakfast.

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*The Tweens (Atlas Powder Co., Wilmington, Del.) are very effective emulsifying agents and are nontoxic when fed to human beings.

Three groups of patients were used for obtaining vitamin A absorption curves. For the first group of absorption curves, eight normal control patients were used. The next group of absorption curves was obtained from eight patients with sprue in relapse. Finally, a third group of observation curves was obtained on six patients who had had sprue but in whom there were no detectable clinical nor laboratory signs of activity and therefore were classified as having sprue in remission.

Tolerance curves on the patients were first obtained with unemulsified vitamin A ester in fish liver oil. Within a week of this original study, another tolerance curve was obtained on the same patients but this time with the feeding of emulsified vitamin A acetate preparation. To each of the three groups of patients was administered the unemulsified vitamin A ester in fish liver oil. To the group of normal patients and to those with sprue in relapse was administered the vitamin A acetate emulsion composed of 15 per cent vitamin A acetate, 15 per cent Tween 20, and 70 per cent water. The test dose in terms of micrograms of vitamin A used in each preparation was the same.

RESULTS

The vitamin A levels as determined by the procedures referred to are compared and summarized in Tables I and II.

TABLE I. COMPARISON OF VITAMIN A ABSORPTION TEST IN NORMAL CONTROLS AND IN PATIENTS WITH SPRUE

(Oleum Percomorphum, 200,000 μg vitamin A per patient, test dose)

CASE	VITAMIN A VALUES IN μG/100 ML. OF PLASMA			
	FASTING	3 HR.	5 HR.	MAX. RISE
Normal Controls				
8 normal controls	Range 40-80	112-280	240-555	
	Av. 60	195	374	314
Sprue in Relapse				
1	58	50	46	00
2	29	31	44	15
3	48	64	100	52
4	65	75	73	10
5	71	134	113	63
6	39	40	72	33
7	10	22	23	13
8	43	49	73	30
Sprue in Remission				
9	51	72	282	231
10	52	58	192	140
11	86	214	405	319
12	64	179	420	356
13	67	91	376	309
14	58	198	356	298

In *normal adult patients* the feeding of unemulsified vitamin A ester in fish liver oil produced significant rises in plasma vitamin A. When the same group of normal patients was given emulsified vitamin A acetate in Tween, a similar but higher peak level in plasma vitamin A was produced. The emulsified material accelerated absorption in only one instance, producing a peak rise at three hours compared to a peak reached in five hours in the remainder of this group.

Adult *patients with active sprue* who were requiring maintenance dosage of folic acid or liver therapy, and all of whom continued to show steatorrhea, were fed unemulsified vitamin A ester in fish liver oil. Very poor absorption

TABLE II. COMPARISON OF VITAMIN A ABSORPTION TEST IN NORMAL CONTROLS AND IN PATIENTS WITH SPRUE: UNEMULSIFIED AND EMULSIFIED MATERIALS

(A, Oleum Percomorphum, 200,000 μg vitamin A per patient, test dose; B, Vitamin A Acetate emulsion, 200,000 μg vitamin A per patient test dose)

CASE AND MATERIAL	VITAMIN A VALUES IN μG/100 ML. OF PLASMA			
	FASTING	3 HR.	5 HR.	MAX. RISE
Normal Controls				
8 normal controls B	Range 40-80 Av. 60	250-640 410	530-980 755	695
Sprue in Relapse				
1 A	58	50	46	00
B	55	432	485	430
2 A	29	31	44	15
B	27	290	340	313
3 A	48	64	100	52
B	57	300	480	427
4 A	65	75	73	10
B	63	710	942	879
5 A	71	134	113	63
B	63	620	946	883
6 A	39	40	72	33
B	56	380	695	639
7 A	10	22	23	13
B	9	240	495	486
8 A	43	49	73	30
B	33	70	115	82

resulted, as may be seen in Table I, the maximum rise of vitamin A being quite low in every patient. However, when preparations of emulsified vitamin A acetate in Tween were fed to this same group, a very marked increase in plasma vitamin A occurred of about the same degree as seen in normal controls. Only one patient, Case 8 in Table II, repeatedly proved an exception and showed very little rise in plasma vitamin A when given emulsified vitamin A acetate in Tween.

When the group of patients classified as having *sprue in remission* were fed unemulsified vitamin A ester in fish liver oil, the results as seen in Table I were, with one exception, fairly comparable to those obtained in the normal controls. After two members of this group were found to have normal absorption curves when fed the emulsified vitamin A material, it was decided not to expend the small supply of this preparation on the whole of the group.

DISCUSSION

Under normal physiologic conditions the absorption curves on adults demonstrate that the tested materials were well absorbed and that the peak levels of plasma vitamin A were somewhat higher following the use of emulsified preparations. Of chief interest were the striking differences in results obtained in patients with sprue in relapse. The marked improvement in vitamin A absorption in these patients, with one exception, was dependent upon the use of emulsified materials. Such emulsions, it has been determined,[1] contain oily droplets varying in size from 0.5 to 10.0 microns. It has been postulated[4] that the degree of the dispersion of fat is perhaps mainly responsible for absorption. Finely dispersed particles result from a suitable emulsifying system. The emulsifying agent, Tween 20, apparently produced particles of appropriate size for enhanced absorption.

Fish liver oil contains vitamin A largely in the form of esters. Therefore, it is thought that the mechanism for its digestion is similar to that of other neutral fats and requires lipolysis. In patients with sprue there is no evidence of defective lipolysis. However, it is to be noted that fish liver oil is *unemulsified* material. Therefore, the hypothesis presented by one investigator,[4] that absorption is mainly dependent upon fine particle size, seems to be further supported by the data herein reported.

The observations on patients with sprue in remission demonstrate that they absorbed the unemulsified material, with one exception, equally as well as normal controls. This would indicate a return to a normal physiologic status of the small intestinal tract. Further evidence of this was found in the normal clinical condition, normal radiologic pattern of the small intestines, and normal quantitative fecal fat.

It should be noted that we do not know the degree of absorption which might result from feeding vitamin A acetate alone. Would this form of vitamin A be as well absorbed without the addition of the emulsifying agent used in these experiments? Since it was impossible to obtain the necessary amounts of vitamin A in this pure form, we cannot answer this question. However, it has been reported[5] that patients with steatorrhea who failed to absorb vitamin A esters in fish liver oil did show a marked increase in vitamin A absorption when polyoxyethylene sorbitan monooleate, "Tween 80," was added to the test dose of the same material.

The implications of these data would seem to be of great practical importance in the management of steatorrhea in sprue. These patients present a difficult problem in dietary fat control. It is probable that their poor absorption of vitamin A, as well as fats, results from a defective emulsifying system in the small intestinal tract. Therefore the ideal diet should probably be one containing vitamin A and fats in emulsified form, or else, more practical, the addition of an emulsifying agent to the diet.

SUMMARY

The feeding of emulsified vitamin A to normal adults resulted in a somewhat higher elevation of plasma vitamin A than did the feeding of unemulsified vitamin A material.

The patients with sprue in remission absorbed unemulsified vitamin A to a normal degree with only one exception.

Patients with active sprue showed very poor absorption when fed unemulsified vitamin A. When these same patients were fed emulsified material, the absorption of vitamin A was increased to approximately normal with one exception.

It is suggested from these results that patients with active sprue require the addition of an emulsifying agent to their dietary fat and fat-soluble substances to promote absorption.

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LABORATORY METHODS

A NEW TABLET TEST FOR URINARY BILIRUBIN

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THE need for a practical solution of the clinical problem of detecting small amounts of urinary bilirubin has received added emphasis with the increased incidence of liver disease during World War II.[1] Until recently we have had no simple, sensitive, and reliable bilirubin test which could be used routinely by the physician for the detection of bile in the urine and which could be employed in the armed forces and industry for mass examinations to detect latent liver disease or follow its course. The standard methods for the detection of bilirubin in the urine, such as the foam, nitric acid, and iodine tests, are relatively insensitive and are difficult to evaluate when pigments other than bilirubin are present in the urine.[2] The methylene blue test,[3-8] although simple and useful as a screening or follow-up test, is nonspecific and may give falsely positive results with urine containing yellow pigments such as riboflavin, penicillin, or Atabrine. It is also less sensitive than other bile tests routinely employed.[9] Tests depending upon concentration of the bile pigment, either by precipitation and adsorption of the pigment by barium chloride or calcium chloride, or upon adsorption by tale are the most sensitive tests.[9] Such tests as the Godfried[10] diazo and Harrison[11] and Naumann[12] spot tests are relatively time consuming. Watson's[1] barium strip modification of the Harrison test has best met the requirements of sensitivity, simplicity, and applicability to mass and serial usage. With this test, higher concentrations of bilirubin give a definite green color. However, at low concentrations of bilirubin (below 0.1 mg. per 100 c.c.) the color becomes blurred and indistinct and we have found the test often to be negative (Table I).

The object of this paper is to describe a simple yet sensitive test which is applicable to clinical work by the physician in everyday practice and to mass screening and serial testing. It is a tablet test, which can be prepared very simply and inexpensively. This test makes use of the principles of concentration of biliary pigment as in the Naumann and Harrison spot tests and subsequent oxidation with resultant color change of the bile pigment by Fouchet's reagent.* Concentration of bilirubin pigment in the Naumann

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*Fouchet's reagent consists of:

Trichloracetic acid	25 Gm.
Distilled water	100 c.c.
10 per cent ferric chloride solution	10 c.c.

TABLE I. SENSITIVITY COMPARISON OF FILTER PAPER TEST AND TABLET TEST FOR BILIRUBINURIA

Urine A

Urinary dilution	0	1/2	1/5	1/10	1/20	1/50	1/100	1/200	1/400	1/600	1/800	Drops of Urine
Concentration* bilirubin (mg. %)	15.7	7.85	3.14	1.57	0.79	0.31	0.15	0.075	0.035	.026		
Filter paper test†	4+	4+	2+	1+	Tr.	Tr.	Sl. Tr.	0	0	0	0	
Tablet test	4+	4+	2+	2+	1+	Tr.	Tr.	Sl. Tr.	0	0	0	10
								Tr.	Sl. Tr.	Sl. Tr.	0	15

Urine B

Urinary dilution	0	1/2	1/5	1/10	1/20	1/50	1/100	1/200	Drops of Urine
Concentration* bilirubin (mg. %)	2.4	1.2	0.48	0.24	0.12	0.048	0.024	0.012	
Filter paper test†	3+	1+	1+	Tr.	Sl. Tr.	0	0	0	
Tablet test	3+	2+	1+	1+	Sl. Tr.	Sl. Tr.	0	0	10
					1+	Tr.	Sl. Tr.	0	15

Urine C

Urinary dilution	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	Drops of Urine
Concentration* bilirubin (mg. %)	1.5	0.75	0.378	0.188	0.094	0.047	0.024	0.012	
Filter paper test†	2+	1+	Tr.	Sl. Tr.	0	0	0	0	
Tablet test	2+	2+	1+	Tr.	Sl. Tr.	Sl. Tr.	0	0	10
					Tr.	Tr.	Sl. Tr.	0	15

Urine D

Urinary dilution	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/236	Drops of Urine
Concentration* bilirubin (mg. %)	6.5	3.75	1.88	0.94	0.47	0.235	0.118	0.059	0	
Filter paper test†	3+	2+	2+	1+	Tr.	Sl. Tr.	0	0	0	
Tablet test	3+	2+	2+	1+	1+	Tr.	Sl. Tr.	0	0	10
							Tr.	Sl. Tr.	0	15

Urine E

Urinary dilution	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	Drops of Urine
Concentration* bilirubin (mg. %)	1.2	0.6	0.3	0.15	0.075				
Filter paper test†	2+	1+	Tr.	Tr.	0	0			
Tablet test	2+	1+	Tr.	Tr.	Sl. Tr.	0			10
					Tr.	0			15

*Concentration of urinary bilirubin determined by Gibson's method.
†Watson's modification of Harrison's spot test.

test is achieved by adsorption to a precipitated emulsion of talc. The Harrison spot test utilizes barium chloride as the adsorbing agent for bilirubin. The tablets used in this test concentrate bilirubin by utilizing the processes of filtration, adsorption by gypsum particles, and barium chloride adsorption. This test is thus a modification of both the Naumann and Harrison spot tests.

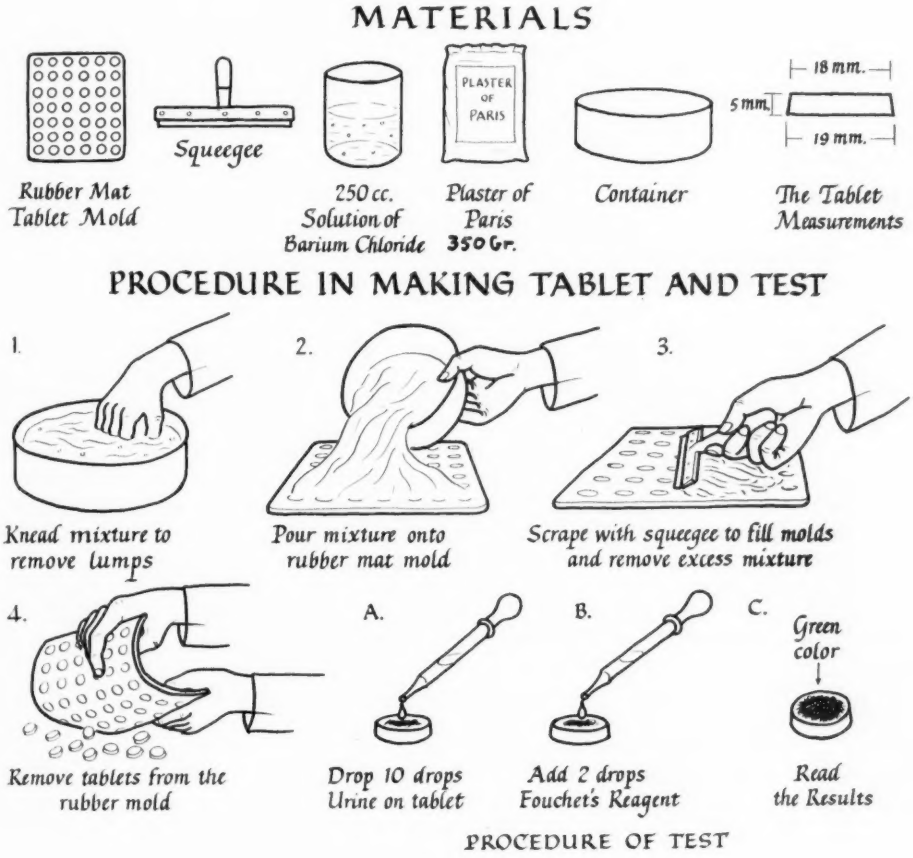

Fig. 1.

DESCRIPTION OF METHOD

A. Preparation of Tablets (Fig. 1, 1-4).—Three hundred fifty grams of ordinary commercial plaster of Paris (not dental plaster) are mixed in a container with 250 c.c. of a saturated solution of barium chloride. The resultant mix is then stirred and kneaded manually to remove any lumpy particles and to produce a homogeneous plaster mix which can be poured. This should be done in less than ninety seconds or the plaster will set in the container. It is then poured into a suitable rubber mold with approximately 250 indentations, each of which is 18 mm. in diameter at the bottom, 19 mm. at the top, and 5 mm. deep. This is the size of the finished tablets. Before pouring the plaster, the rubber mat should be moistened by placing it under a running faucet or dipping it in water. The mat is inverted to get rid of excess water and the plaster is then poured onto the moistened mat. A squeegee (used to clean windows) is then drawn over the surface of the mat several times, distributing the wet plaster evenly in the indentations. The plaster is allowed to become firm and after

†Watson's modification of Harrison's spot test.

fifteen minutes the mat is inverted and the firm but still moist tablets will drop out. These are then dried in an oven and are ready for use at any time thereafter. The entire procedure of preparing the tablets until they are ready for drying should not take more than twenty minutes.

With the described proportions of plaster of Paris and barium chloride solution, the tablets should be of the proper consistency and should give optimal results. The proportion of 1.4 Gm. of plaster of Paris for every 1 c.c. of saturated barium chloride may have to be altered somewhat, depending upon the brand of plaster of Paris used. If the resultant tablets are too hard and do not absorb the urine quickly enough, the proportion of plaster of Paris to the saturated barium chloride may be decreased. Ordinary tap water instead of the saturated barium chloride solution may be used in the preparation of the tablet. However, tablets prepared in this manner do not adsorb as much bilirubin, making the test less sensitive. The rubber mold we have used is an ordinary commercial door mat,* 1 ft. by 2 ft., containing 500 indentations. The upper ridged surface is made smooth with sandpaper and the mat is cut in two, one-half of this mat being used because it is easier to work with a smaller mold. An improved mat could be prepared with indentations of proper size, but with the bottom surface convex so that the prepared tablet would have a concave rather than a flat surface. The urine could then be dropped onto the concave surface of the indented tablet.

B. Procedure (Fig. 1, A, B, C).—Using an ordinary eye dropper or a pipette measuring 20 drops to the cubic centimeter, 10 drops of urine are dropped slowly upon the surface of the tablet so as not to cause any overflow. If bilirubin is present in the urine, a fine layer of yellow bile pigment will be adsorbed upon the surface of the tablet. The remainder of the urine will filter through. One or two drops of Fouchet's reagent are then dropped directly on the yellow area. A positive test is denoted by a blue-green color, which varies in intensity with the amount of bilirubin present. The test is read in the same manner as the Harrison strip test: trace to 4 plus. However, with urine containing low concentrations of bilirubin, the color change is much sharper with the tablet test. Normal urinary pigments give neither the yellowish discoloration on the tablet surface nor a positive test with the use of Fouchet's reagent. After addition of Fouchet's reagent, the green color should be read immediately, or its intensity will decrease. When no positive test is secured after addition of Fouchet's reagent, and it is still suspected that there might be a very faint trace of bile present, the use of 15 drops instead of 10 (the tablet will usually become flooded with more than 15 to 20 drops) may bring out faint traces of bile. This can be seen as a faint yellowish discoloration and will give a very faint but perceptible bluish-green color after the addition of Fouchet's reagent. Occasionally in concentrated normal urines, as with the Watson modification of Harrison's strip test, a gray or lavender color develops after the addition of Fouchet's reagent. This is not due to bile pigment, but probably indicates dehydrogenation products of urobilinogen.[13]

DISCUSSION

This tablet test has been performed on more than 1,000 urine specimens. There have been no false positive results. In many instances it has been compared simultaneously with other urine tests, such as the foam, nitric acid, iodine, methylene blue, and Watson's modification of the Harrison spot test (Table I). It is inexpensive to prepare, simple to perform, more sensitive and easier to read than any of the afore-mentioned tests. We have obtained a positive test with urines containing as little as .025 mg. bilirubin, although at these very low concentrations the test becomes difficult to read. We are attempting at present to correlate the degree of positivity of the test with bilirubin levels in the blood.

*Rubber Maid Door Mat, manufactured by the Wooster Rubber Company, Wooster, Ohio.

Ordinary urine pigments give no yellowish color on the tablet surface nor any bluish-green color after Fouchet's reagent is added. Certain therapeutic agents, such as riboflavin, Atabrine, and Pyridium which discolor urine, may give a slight yellowish discoloration of the surface of the tablet. The first two, however, give no such discoloration with ordinary therapeutic doses. None of these drug pigments give a positive test after the addition of Fouchet's reagent. Urine from patients with hematuria will also discolor the surface of the tablet, but no green color is produced after the addition of Fouchet's reagent. We have noted a purplish-black pigment adsorbed onto the tablet surface from the urine of a patient with acute porphyria. This tablet when held under a Wood's ultraviolet lamp gave the red fluorescence of a porphyrin. When bile pigment is present with any of these extraneous pigments a positive test is still secured with Fouchet's reagent, although it is somewhat altered by the presence of these other pigments. The intensity of the yellowish color on the tablet surface or the green surface or the green color after the addition of Fouchet's reagent can be utilized to determine semiquantitatively the amount of bile pigment in the urine, as Watson[13] has done with the Harrison strip test. The yellow color can be used provided the patient has not been given any Atabrine, riboflavin, or Pyridium. With the therapeutic doses of Atabrine or riboflavin used routinely, the urinary excretion of these drugs does not discolor the surface of the tablet to any noticeable degree. However, none of the drugs interfere with the green color obtained after the addition of Fouchet's reagent. A urine containing bile can be analyzed by a quantitative method such as the Gibson* method or the Godfried[10] modification of the Hunter diazo method. Various dilutions can be made and the test run upon these different dilutions of the urine of known bilirubin pigment concentration. Either the initial yellow color or the green color after the addition of Fouchet's reagent can be used to determine the quantity of bilirubin. Color photographs or drawings can be made of these different intensities which can then serve as standards for a given batch of tablets.

The formula for plaster of Paris is $Ca\ SO_4 . \frac{1}{2}H_2O$. After addition of water the dry plaster sets to a coherent solid which is composed of interlacing crystals of gypsum ($Ca\ SO_4 . 2\ H_2O$).[14] When urine containing bile is dropped upon the tablet surface, the gypsum particles apparently adsorb the pigment, as does talc in the Naumann test. There may be an added chemical adsorptive effect because of the presence of calcium in the gypsum molecule. Addition of barium chloride in the preparation of the tablet definitely increases the

*Gibson's method for quantitative urinary bilirubin:
Barium acetate 0.25 Gm. is introduced into a 15 ml. centrifuge tube, and 10 ml. of urine (pH 5 to 6) are poured into it and the tube is thoroughly shaken. The tube is centrifuged and after fifteen minutes the supernatant liquid is poured off, and the wall of the tube and the precipitate are washed gently with water and the tube is drained. One milliliter of Ehrlich's diazo reagent is added and the precipitate is thoroughly stirred with a glass rod. Two milliliters of water, 2 ml. of saturated ammonium sulfate solution (again stirring), 8 ml. of alcohol, and 2 ml. of concentrated hydrochloric acid are then added. Let stand with occasional shaking and filter (Whatman No. 5 paper). Read in a Coleman Junior spectrophotometer in an 85 by 12 mm. cuvette, wave length 575, with a water reference. If too strong, the filtrate may be diluted appropriately with a blank solution. The graph secured with the spectrophotometer on semilogarithmic paper is a straight line from 0 mg. per cent at 100 per cent transmission to 1 mg. per cent at 24 per cent transmission when 10 ml. of urine are precipitated.

bilirubin adsorption. The addition of Fouchet's reagent oxidizes the bilirubin to green biliverdin. This bluish-green color tends to fade and become less intense after several minutes, especially with urine containing very small amounts of bile. The principle of using a plaster of Paris tablet as an adsorbing filter could be applied to other substances such as porphyrin and melanin. We are at present investigating this possibility.

SUMMARY

1. The preparation and use of an inexpensive, simple, sensitive table test for detection of urinary bilirubin are described. This test is a modification of Naumann and Harrison spot tests for bilirubin.

2. The test makes use of a plaster of Paris tablet, made with a solution of saturated barium chloride or tap water, upon the surface of which urine is dropped. The addition of Fouchet's reagent to the bilirubin pigment which is adsorbed on the tablet surface gives a clear-cut, easily readable, bluish-green color which is specific for bile. The test can be used for mass and serial testing and can be made semiquantitative for the determination of urinary bilirubin.

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TECHNIQUES TO OVERCOME THE LACK OF RARE RHESUS ANTISERA AND CELLS*

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COMPARATIVELY few laboratories can rely on obtaining supplies of the rarer Rhesus antisera such as pure anti-C *(anti-rh')* and pure anti-E *(anti-rh'')*, nor are donors of the rarer blood cells Cde/cde or Cde/Cde *(rh')* and cdE/cde or cdE/cdE *(rh'')* readily available. Most laboratories undertaking Rh typing will, however, have supplies of the mixed antisera C + D *(anti-Rh_0')* and D + E *(anti-Rh_0'')* as well as complete and incomplete pure anti-D *(anti-Rh_0)*.

The preparation of anti-C *(anti-rh')* and anti-E *(anti-rh'')* from mixed sera is often unsatisfactory. Dilution removes the anti-C and anti-E, which are almost invariably of lower titer than the anti-D *(anti-Rh_0)*, and when an absorption technique is employed, the titer of the anti-C and anti-E, already low as a rule, is still further reduced.

The lack of the rare cells, Cde/cde or Cde/Cde *(rh')* and cdE/cde or cdE/cdE *(rh'')*, prevents the testing of sera for anti-C or anti-E in the presence of anti-D, the sera having to be stored until these cells are available, while the lack of pure anti-C *(anti-rh')* or anti-E *(anti-rh'')* causes difficulties in genotyping.

Techniques are described below which overcome these difficulties. They have been used in this laboratory with satisfactory results, having been controlled by the use of pure anti-C *(anti-rh')* and anti-E *(anti-rh'')* and by fresh cells of the types Cde/cde and cdE/cdE *(r'r* and *r''r'')*.

The principle on which they are based is the exclusion of the D antigen *(R^0)* in CDe/cde *(R^1r)* or cDE/cde *(R^2r)* cells and the D *(R^0)* antibody in mixed anti-C + D *(anti-Rh_0')* or anti-D + E *(anti-R_0'')* from the field of action by means of a strong incomplete anti-D *(anti-Rh_0)* serum using a modification of the blocking technique originally described by Wiener.[1]

Detection of the Antibodies C and E *(anti-rh'* and *anti-rh'')* in the Presence of Anti-D *(anti-Rh_0)* by Means of Cells of the Common Genotypes CDe/cde and cDE/cde *(R^1r* and *R^2r)*.—

"Prepare" cells of the comparatively rare genotypes Cde/cde *(r'r)* and cdE/cde *(r''r)* from cells of the common genotypes CDe/cde *(R^1r)* and cDE/cde *(R^2r)* as follows:

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*At the present time, two systems of nomenclature of the anti-Rh typing sera and cells are in wide use.[3, 4, 5] One of the systems is based on the series of allelic genes proposed by Wiener,[3] the other on a series of closely linked pairs of genes proposed by Race and Fisher of England.[5] A comparison of the individual antiserums has been made recently by the advisory board of the National Institute of Health.[4] In this paper, the Race-Fisher nomenclature has been employed, but for the convenience of the reader, Wiener's nomenclature is also given in parentheses and italics.

1. Subject a saline suspension of the cells CDe/cde *(R^1r)* or cDE/cde *(R^2r)* to the action of a strong incomplete (blocking) pure anti-D *(anti-Rh_o)* serum for one and one-half to two hours at 37° C.

2. Examine for absence of agglutination and confirm "coating" by means of Coombs reagent (antihuman globulin serum).

These "prepared" cells can now be used to detect the presence or absence of anti-C *(anti-rh′)* or anti-E *(anti-rh″)* in a serum known to contain anti-D *(anti-Rh_o)*.

3. Divide the cell suspension into three portions—one test and two controls.

Test one with the serum under examination, incubating for one and one-half to two hours at 37° C.

Agglutination indicates the presence and no agglutination the absence of anti-C *(anti-rh′)* or anti-E *(anti-rh″)*.

Test two with a saline agglutinating anti-D *(anti-Rh_o)* serum having a titer comparable with that of the anti-D in the mixed serum, incubating for one and one-half to two hours at 37° C.

Incubate the third at the same time as one and two.

No agglutination should occur in two and three.

Detection of the Antigens C *(r′)* and E *(r″)* in the Presence of D *(R^o)* by Means of Mixed Anti-C + D *(Anti-Rh_o′)* and Anti-D + E *(Anti-Rh_o″)* Sera.—

1. Subject a saline suspension of the cells under examination to the action of an incomplete (blocking) pure anti-D *(anti-Rh_o)* serum for one and one-half to two hours at 37° C.

2. Examine for absence of agglutination and confirm "coating" by means of Coombs reagent.

These "prepared" cells are now inagglutinable by anti-D *(anti-Rh_o)*, and mixed anti-C + D *(anti-Rh_o′)* or anti-D + E *(anti-Rh_o″)* serum can be used to detect in them the antigen C *(r′)* or E *(r″)*.

3. Divide the cell suspension into three portions—one test and two controls.

Test one with the mixed anti-C + D *(anti-Rh_o′)* or anti-D + E *(anti-Rh_o″)* serum, incubating for one and one-half to two hours at 37° C.

Agglutination indicates the presence and no agglutination the absence of the antigens C *(r′)* or E *(r″)*.

Test two with a saline agglutinating anti-D *(anti-Rh_o)* serum having a titer comparable with that of the anti-D in the mixed serum, incubating one and one-half to two hours at 37° C.

Incubate the third at the same time as one and two.

No agglutination should occur in two and three.

Note.—Where cells of the genotype Cde/cde *(r′r)* or cdE/cde *(r″r)* are available, attention should be drawn to the fact that a proportion of them are CD^ue/cde or cD^uE/cde (Stratton).[2] A preliminary investigation in this laboratory shows that about 10 per cent of so-called Cde/cde *(r′r)* cells are CD^ue/cde. The technique outlined herein for the "preparation" of cells will

prevent errors through using a CD^{u}e/cde cells thought to be Cde/cde *(r'r)* or a cD^{u}E/cde cell thought to be cdE/cde *(r''r)*.

Wiener's nomenclature[3, 4, 5] is given in parentheses and italics for the convenience of those laboratories which still adhere to it, but difficulty is found in expressing the various allelomorphs of D, C, and c which are now known to exist, e.g., in this paper no equivalent in Wiener's nomenclature is suggested for the cells CD^{u}e and cD^{u}E: this may be left to the ingenuity of the reader.

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A GALVANOTACTIC PROCEDURE FOR THE CONCENTRATION OF BALANTIDIUM COLI IN FECES

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INTRODUCTION

THE diagnosis of balantidiasis is seldom established by physicians because the disease is little known. Another important reason is that *Balantidium coli* are seldom found in fresh stool specimens. The common helminthological and parasitological methods of stool examination utilize only a small quantity of fresh material and often kill or injure the balantidia. By examining stool specimens diluted with physiologic saline, Stschensnowitsch in Azerbaijan[5] was able to increase materially the percentage of positive results. He found *B. coli* in 56 (5.1 per cent) of 1,100 cases. Before this study, only four cases of balantidiasis were discovered in the whole of Azerbaijan.

The employment of a procedure which would increase the recovery of parasites from stools would be of value in the diagnosis of balantidiasis. The idea of concentrating the parasites by employing the phenomenon of galvanotaxis used in the study of free-living Infusoria and of parasitic Infusoria of frogs[1-6] suggested itself. The apparatus, which utilizes a galvanic current and is described below, has been successfully used for concentrating Infusoria from the rectum of the frog and *b. coli* from the feces of swine.

MATERIALS AND METHODS

Apparatus.—As a source of current, a Kenotron rectifier with a potentiometer was used, but any standard rectifier is suitable. The apparatus produced a direct current of 132 volts. Preliminary investigations were set up in a chambered slide. These chambers were constructed with glass strips 2 mm. thick and 5 mm. wide and sealed with Canada balsam in such a way that three chambers of not quite equal size resulted (Fig. 1). These chambers were connected by means of channels 5 by 5 by 2 mm. in size. The channels were initially filled with 2.5 per cent agar (pH 7.5) in Ringer's solution. The central chamber was filled with a balantidia-containing fecal suspension in Ringer's solution and covered with a cover glass. The side chambers were half filled with Ringer's solution in which were immersed pipette-like agar bridges connecting them with Petri dishes containing Ringer's solution. The Petri dishes were connected by means of agar bridges with other dishes containing saturated copper sulfate solution in which were immersed copper electrodes. The resistance between the electrodes was approximately 30,000 to 35,000 ohms. The diffusion of copper ions into the chamber may be practically eliminated by changing the Ringer's solution in the dishes at one-half to one-hour intervals.

Warming the chamber to 37 to 42° C. facilitated the collection of balantidia at the cathode. Therefore, in the construction of a practical apparatus, a means of warming the chamber as well as increasing its volume had to be considered.

As concentration vessels, stool sample tubes 70 mm. in length and 22 mm. in diameter were chosen. These are immersed in a water bath in such a manner that they project a distance of about 1 cm. over the cover plate, a rubber ring being placed about the tube

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at this level. The anodic agar bridge was dipped a few millimeters into the suspension. The cathodic bridge is inserted into the upper, dilated end of a special concentration tube, suspended in the stool sample tube by a glass hook (Figs. 2 and 3). The lower end of the concentration tube is closed by a porcelain filter and the tube filled with Ringer's solution.

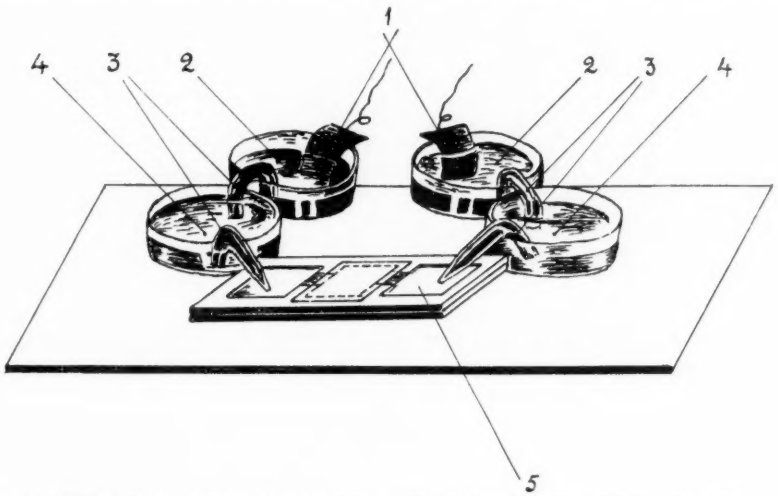

Fig. 1.—Chamber apparatus for concentrating Infusoria from rectum of frog.
1, Copper electrodes.
2, $CuSO_4$ solution.
3, Agar bridges.
4, Ringer's solution.
5, Galvanotactic chamber.

A short length of rubber tubing is placed about the end of the tube which does not reach the bottom of the stool sample tube. The balantidia collect inside the lumen of the rubber tube below the porcelain filter (Figs. 2 and 3).

In filling the concentration tube and rubber tube with Ringer's solution, no air bubbles must be permitted to enter, since these greatly increase electrical resistance. A cellophane cap may be used in place of the porcelain filter. If cellophane is used, one must be sure that no portion protrudes beyond the rubber tubing, since balantidia would also collect there. Instead of the concentratoin tube described, one may use a long cathodic agar bridge which is filled with agar, except for about 5 mm. at the lower end, this space being filled with Ringer's solution. Such an agar bridge may be used only once, since the balantidia migrate into the capillary space between the wall of the tube and the agar. The concentration tube equipped with the porcelain filter need only be immersed in boiling water before reusing. The concentration process was carried on for a period of ten minutes at 39° C. The resistance when the porcelain filter was used was about 35,000 to 40,000 ohms, with cellophane, 20,000 to 25,000 ohms. The current strength was 0.3 to 6.0 milliamperes.

Then the concentration tube was removed from the stool sample tube, and the contents of the concentration space (below the porcelain plate) were pipetted out and examined microscopically.

Staining Specimens.—For counting the *B. coli*, a small drop of saturated potassium iodide-iodine solution was added. For nuclear staining, a mixture of equal parts of a saturated aqueous solution of methyl green and 40 per cent formaldehyde (formalin) was employed.

In our material (cecal contents of swine) balantidia of the suis type with long, sausage-shaped nuclei predominated. Balantidia with round or bean-shaped nuclei were seen only in about 15 per cent of swine. They formed less than 1 per cent of the whole balantidial population. The stool specimens were examined between two and six hours after the swine were killed.

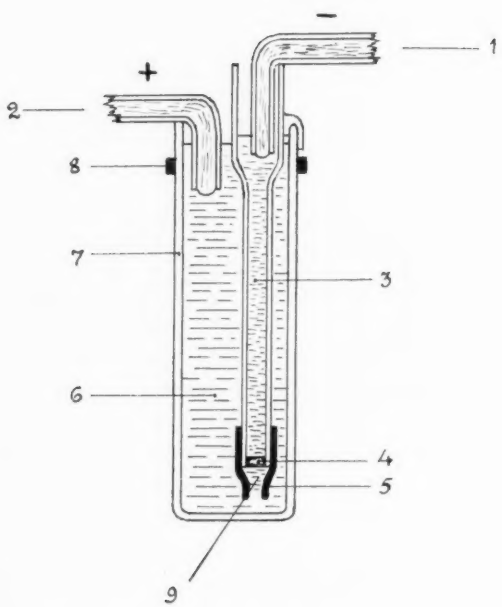

Fig. 2.—Concentration tube for collecting *B. coli.*
1, Cathodic agar bridge.
2, Anodic agar bridge.
3, Concentration tube.
4, Porcelain filter.
5, Rubber tubing.
6, Suspension of feces in Ringer's solution.
7, Test tube.
8, Rubber ring.
9, Concentration space ("trap").

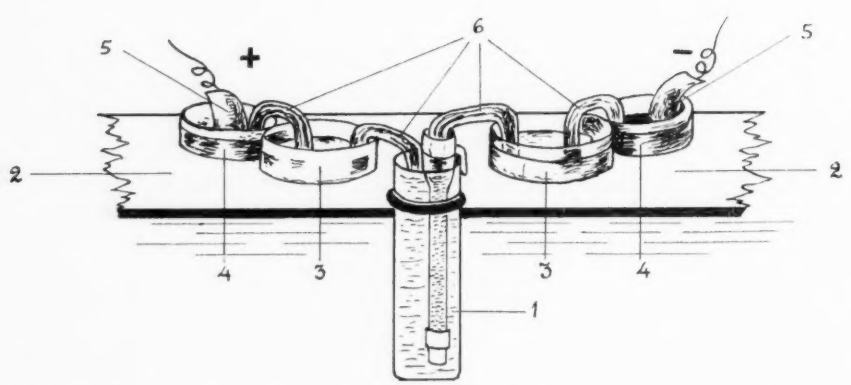

Fig. 3.—Concentration tube with electrical connection for collection of *B. coli* from swine feces.
1, Test tube.
2, Lid of the water bath.
3, Petri dishes with Ringer's solution.
4, Petri dishes with copper sulfate solution.
5, Copper electrodes.
6, Agar bridges.

The preliminary observations employing the apparatus shown in Fig. 1 were done using Infusoria from the frog rectum and swine balantidia. *Balantidium entozoon* (frog) and also the swine balantidia collected at the cathode at room temperature, with a current strength of 0.3 to 1.0 Ma. for three to five minutes, and remained there as long as the current was flowing. Under the same conditions, *Opalina ranarum* and *Opalina dimidiata* collected at the anode, where they soon were killed. Apparently it is possible to separate single species of ciliophoria from a mixed population in establishing the necessary current strength for the anadromus movement of different species.

The stool specimens taken from swine ceca were first investigated without concentrating and four cover glass preparations of sediment of a fecal suspension in a Ringer's solution (1:3) were examined at low magnification. Then the galvanotactic chamber (Fig. 1) was filled with the same sediment and the inside surface of the agar cathode was examined at low magnification. Of twenty stool specimens investigated in this manner, balantidia were found without concentration in eight specimens, while with concentration they were found in twelve specimens. The percentage of positive results was increased therefore only from 45 to 60 per cent, which was to be expected, since the volume of the chamber (0.45 ml.) is hardly greater (Fig. 1) than the sediment observed under the four cover glasses.

Optimal Position of the Electrodes.—Fifteen milliliters of a 1 to 3 dilution of stool suspension were placed in each of two sample tubes (Fig. 2) and concentrated for ten minutes at 39° C. using a current of 3 milliamperes. With one of the samples the long cathodic concentration tube described was used. With the second sample a long anodic agar bridge was employed with a short cathodic concentration tube which extended only 1 cm. beneath the surface. In Table I are shown the numbers of balantidia "captured" at different electrode positions.

TABLE I. POSITION OF ELECTRODES IN RELATION TO RECOVERY OF B. COLI

EXPERIMENT	WITHOUT CONCENTRATION*	WITH CONCENTRATION	
		SHORT CATHODE	LONG CATHODE
1	0.25	0	0
2	3.75	92	174
3	1.75	23	98
4	0.75	1	45
5	0	0	24
6	0	2	17

*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

The best results were obtained when the cathode was near the bottom of the stool sample tube, a fact probably dependent on the positive geotaxis of balantidia.

Optimal Temperature.—Each stool suspension was divided into six equal portions and concentrated at temperatures ranging from 25° to 50° C., with a current strength of 3 Ma. for ten minutes. The results are shown in Table II.

TABLE II. TEMPERATURE OF FECES SUSPENSION IN RELATION TO RECOVERY OF B. COLI

EXPERIMENT	WITHOUT CONCENTRATION*	WITH CONCENTRATION					
		25° C.	30° C.	35° C.	40° C.	45° C.	50° C.
1	3.5	2	14	73	126	83	0
2	2.5	2	9	8	100	15	0
3	0	0	2	4	4	0	0
4	0.75	8	31	115	135	14	0
5	1.0	0	5	17	52	43	0

*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

The optimal temperature is 40° C., as Table II shows. At 25°, the balantidia are not concentrated by this apparatus and between 45° to 50° the Infusoria died. A temperature of 39° C. was thereafter chosen for our experiments, corresponding to the rectal temperature of swine.

Duration of Concentration.—In order to determine the most practical time interval for concentrating the balantidia, experiments were done lasting eighty minutes at a temperature of 39° C., with a current strength of 1 milliampere. Every ten minutes the concentration tube was removed and the balantidia in the trap were counted. The results are given in Table III.

TABLE III. TIME NECESSARY FOR MAXIMUM CONCENTRATION OF B. COLI

EXPERIMENT	WITHOUT CONCENTRATION*	NUMBER OF BALANTIDIA RECOVERED AT 10-MINUTE INTERVALS WITH CONCENTRATION							
		1	2	3	4	5	6	7	8
1	0	18	15	4	1	–	–	–	–
2	4.0	1,732	168	85	53	38	27	51	22
3	3.0	938	105	54	29	11	6	5	6
4	0.5	35	14	11	5	2	–	–	–
5	0	3	–	–	–	–	–	–	–

*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

As is shown in Table III, about 50 to 80 per cent of balantidia were obtained in the first ten minutes. The number of balantidia diminished rapidly in later samples, although at the end of eighty minutes some still were present in two samples. Therefore, if no balantidia are obtained in the first ten minutes, further stool suspension should be investigated. Similar results were obtained when a current of 3 Ma. was used.

Optimal Current Strength and Its Relationship to the Age of the Stool Sample.—In determining the optimal amperage, four tubes were filled with stool suspension and currents of varying intensity (0.3, 1.0, 3.0, and 6.0 Ma.) were passed for ten minutes at 39° C. The optimal current strength for concentrating balantidia from fresh stool samples was mostly between 1 and 3 milliamperes. In older stool samples, optimal current strength varies so that we were obliged to repeat our experiments until balantidia could no longer be found. They were found to persist in stool samples a maximum of ten days at room temperature. This is in contradiction to usually accepted ideas. Table IV shows the results.

TABLE IV. OPTIMAL CURRENT STRENGTH AND AGE OF FECES SAMPLE IN RELATION TO RECOVERY OF BALANTIDIA

ROOM TEMPERATURE 22°-26° C.

AGE (DAY)	STOOL SAMPLE 85			STOOL SAMPLE 87			STOOL SAMPLE 88		STOOL SAMPLE 89		
	1	2	3	1	2	3	1	2	1	2	3
Without concentration*	2.5	0	0	2.75	0	0	0	0	1.25	0	0
0.3 Ma.	27	5	0	252	0	0	1	0	13	0	0
1.0 Ma.	54	18	0	355	4	0	5	0	48	5	0
3.0 Ma.	57	35	0	372	11	0	1	0	69	5	0
6.0 Ma.	30	11	0	299	10	0	1	0	174	14	0

AGE (DAYS)	STOOL SAMPLE 90		STOOL SAMPLE 91		STOOL SAMPLE 92		STOOL SAMPLE 93		STOOL SAMPLE 94	
	1	2	1	2	1	2	1	2	1	2
Without concentration*	0	0	0	0	0	0	2.25	0	0	0
0.3 Ma.	0	0	0	0	0	0	15	0	0	0
1.0 Ma.	0	0	0	0	0	0	150	0	2	0
3.0 Ma.	1	0	1	0	0	0	97	0	1	0
6.0 Ma.	0	0	0	0	2	0	62	0	2	0

AGE (DAYS)	STOOL SAMPLE 86									
	1	2	3	4	5	6	7	8	9	10
Without concentration*	40.5	3.25	4.0	1.0	0.25	1.0	1.75	1.0	0	0
0.3 Ma.	2,839	42	97	6	3	0	2	1	2	0
1.0 Ma.	3,639	389	86	50	24	6	1	6	0	0
3.0 Ma.	3,803	490	84	125	107	23	41	11	2	0
6.0 Ma.	2,479	547	322	280	53	51	52	2	0	0

*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

It is apparent that balantidia remain viable up to ten days. In Fig. 4 the percentage of positive findings after concentration are plotted against the age of the stool sample. All twenty-five samples were positive on the first day. The disappearance of balantidia from the stool samples does not depend wholly on a change in pH. They may disappear even in neutral samples (pH 7.0 to 7.2). If numerous starch granules are present, they may sometimes be found in acid stools (up to pH 5.6). When the concentrating procedure was used, balantidia could frequently be demonstrated one day later than without concentration.

The optimal current strengths tend to increase with the age of the stool sample. After several days, this amounts to 3 to 6 milliamperes. Such amperages are, however, also optimal for some fresh stool samples containing only few balantidia. The effect of pH on the optimal current strength must be further investigated.

We have examined one hundred stool samples from swine ceca, at a temperature of 39° C., current strength 3 Ma., for ten minutes. Without concentration, 56 per cent were positive, with concentration, 92 per cent were positive. The results of twenty of these experiments are shown in Table V.

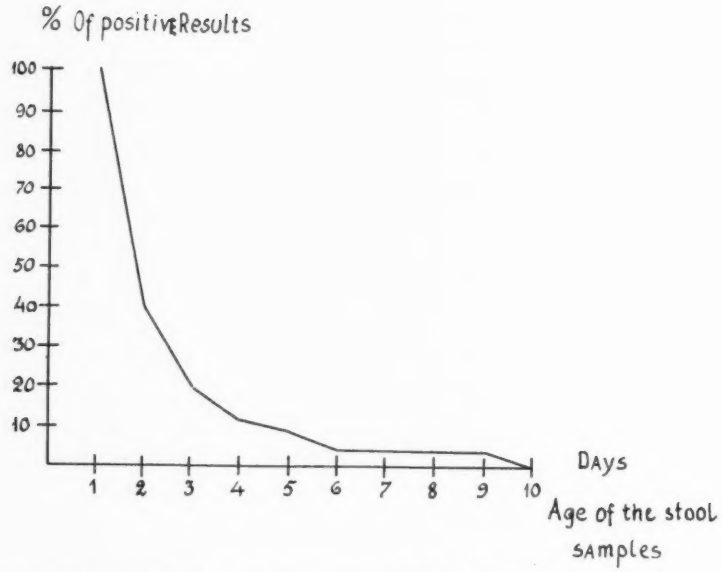

Fig. 4.—The recovery of *B. coli* in relation to age of stool samples. All twenty-five samples were positive on the first day.

TABLE V. NUMBER OF B. COLI OBSERVED WITH AND WITHOUT CONCENTRATION IN TWENTY DIFFERENT SAMPLES OF FECES

NUMBER OF BALANTIDIA			NUMBER OF BALANTIDIA		
SAMPLE	WITHOUT CONCENTRATION*	WITH CONCENTRATION	SAMPLE	WITHOUT CONCENTRATION*	WITH CONCENTRATION
1	4.25	186	11	4.0	1,732
2	99.75	3,927	12	3.75	57
3	6.75	354	13	0.5	9
4	41.0	4,230	14	0	3
5	9.0	299	15	0	0
6	1.25	52	16	5.25	436
7	11.5	4,430	17	0	10
8	26.25	3,312	18	2.5	100
9	2.5	84	19	0	4
10	0	18	20	0.75	112

*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

It is seen that the number of balantidia after concentration is not directly proportional to the number obtained without concentration. For example, in Sample 13 the increase was eighteen fold, while in Sample 11, it was four hundred twenty fold. It is possible that these results might be improved by taking into consideration other factors, such as pH.

On examining fifty stool specimens from swine from another region, the positive results were increased from 34 per cent to 52 per cent by using the concentration procedure.

SUMMARY AND CONCLUSIONS

1. A galvanotactic method for concentrating *B. coli* from swine feces has been described.

2. By using this concentration procedure, the percentage of positive results has been substantially increased from 56 per cent to 92 per cent in one hundred samples and from 34 per cent to 52 per cent in fifty samples from swine from a different locality.

3. It is suggested that this galvanotactic method also may be employed for concentrating *B. coli* and other protozoan parasites in the feces of man.

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THE CADMIUM REACTION

A Practical Test for the Evaluation of Serum Lability—Comparison With the Cephalin-Cholesterol Flocculation and Thymol Turbidity Test

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A NUMBER of publications dealing with the application of Hanger's test (cephalin-cholesterol flocculation test 1938) and that of Maclagan (thymol turbidity test, 1944) indicate that these procedures are being employed to an increasing extent in the clinicochemical laboratory as nonspecific serum reactions. However, the colloidal nature of both reagents in these reactions made it inevitable that many investigators should report difficulties caused by the labile dispersion of these reagents (Frisch and Quilligan,[1] 1946; Wade and Richman,[2] 1945; Dick,[3] 1945). We therefore made it our aim to develop a simple lability reaction whose truly dissolved reagent may be kept indefinitely yet be reproduced easily and uniformly. As we reported in 1945 (Wunderly and Wuhrmann[4]) a 0.4 per cent solution of cadmium sulfate fulfills these requirements.

TECHNIQUE

In a small test tube is placed 0.4 c.c. of the freshly centrifuged serum of a *fasting* patient. Four drops (0.2 c.c.) of 0.4 per cent solution of $3CdSO_4 . 8H_2O$ are then added slowly from a precision pipette. (It has been our custom to use a pipette so dimensioned that twenty freely falling drops of distilled water at 15 degrees attain a weight of 1 Gm. ± 0.05.) During the addition the mixture is shaken gently to insure mixing. The tube is examined immediately, again after one minute, and after five minutes, and if turbidity has appeared, that fact and the degree of such turbidity is noted. For measuring the latter it has proved of advantage to view the tube with a window as background; if turbidity is intense enough after five minutes to cause the crossbar of the window to disappear, the reaction is designated as positive (+ to ++). If turbidity is already developed at one minute, or after addition of two drops of cadmium sulfate solution, such serums are classified as strongly positive. Slight, though still clearly perceptible, turbidity is designated as ±. Normal serum of fasting subjects never becomes turbid. In order to obtain comparable results test conditions must be exactly fulfilled.

COMPARISON OF THE REACTION MECHANISM WITH THAT OF THE THYMOL AND CEPHALIN REAGENTS

Since the cadmium reagent is a simple salt solution, whereas the thymol and cephalin reagents are complexes of several substances, it could be foreseen that the method of action toward the serum components would vary. We have already dealt in detail with the individual factors (Wunderly and Wuhrmann,[5] 1947) and would merely mention that in reactions with colloid-disperse reaction partners it is best to allow only a short interval to elapse before reading, since complex processes such as ageing and ripening are apt to take

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place and make it difficult to obtain comparable results. Much the same considerations apply to the thymol and cephalin-cholesterol reagents themselves. Their limited miscibility with water tends to decrease their dispersion. Repeated checking of turbidity with the Zeiss step-photometer has shown us that the reagents mentioned alter to such an extent that they cannot be employed for more than five days. In contrast, 0.4 per cent cadmium sulfate solution may be kept for an unlimited time.

In order to ascertain which protein subfractions combine with the cadmium sulfate, sufficient cadmium reagent was added drop by drop to 6.5 c.c. plasma samples (taken from patients in whom the usual cadmium reaction has been positive) to produce a considerable amount of precipitate after the usual five-minute standing period. The precipitate was separated by centrifuging, and the serum was then decanted. It was dialyzed against physiologic salt solution in the refrigerator for forty-eight hours, and then again in the usual manner against Michaelis' HCl-Veronal-Na-acetate buffer of 7.9 pH and μ of 0.1. Subsequent electrophoresis gave quantitative information concerning the proteins precipitated. (Table I.) Difficulty from the boundary differences was avoided by the pretreatment. The selected sera had an extremely irregular protein composition caused by disease and showed strongly positive cadmium reactions.

TABLE I. $CdSO_4$-PRECIPITATION OF PROTEINS IN THE SERUM OF THREE PATIENTS

SERUM			1	2	3
DIAGNOSIS			CHRONIC POLYARTHRITIS	PULMONARY TUBERCULOSIS	GAMMA GLOBULIN MYELOMA
Quantity of serum	(c.c.)		6.5	6.5	6.5
Protein content of serum	(Gm. %)		5.3	4.7	13.5
$CdSO_4$-reagent containing $3CdSO_4 8H_2O$	ml.		3.0	3.25	2.5
	mg.		12	13	10
Composition of the native serum in %	Albumin		32.0	21.9	11.5
	Globulin	α	9.8	31.4	3.9
		β	18.3	24.0	8.4
		γ	39.9	22.7	76.2
Decrease of protein by the precipitation with $CdSO_4$ in	(Gm. %)		1.5	1.4	5.4
The same in % of serum-protein			30.2	29.8	40.0
Decrease in % of the protein-fractions after precipitation of $CdSO_4$	Albumin		38.8	18.6	6.1
	Globulin	α	2.6	24.2	2.2
		β	11.2	24.2	5.1
		γ	47.4	33.0	86.6

Table I shows that in Serum 3 (gamma globulin myeloma) 2.5 ml. of the $CdSO_4$ reagent were sufficient to precipitate 5.4 Gm. per cent of the serum albumin content, whereas in Serum 2 (exudative pulmonary tuberculosis) only 1.4 Gm. per cent were precipitated with 3.25 milliliters. In Serum 1 (chronic rheumatic polyarthritis; for electrophoretic diagram see Wunderly and Wuhrmann, Experientia II/8, 1946, Switzerland), where the albumin content was relatively the largest (32 per cent), the greatest quantity of albumin was also precipitated, viz., 38.8 per cent of the total 1.5 Gm. per cent.

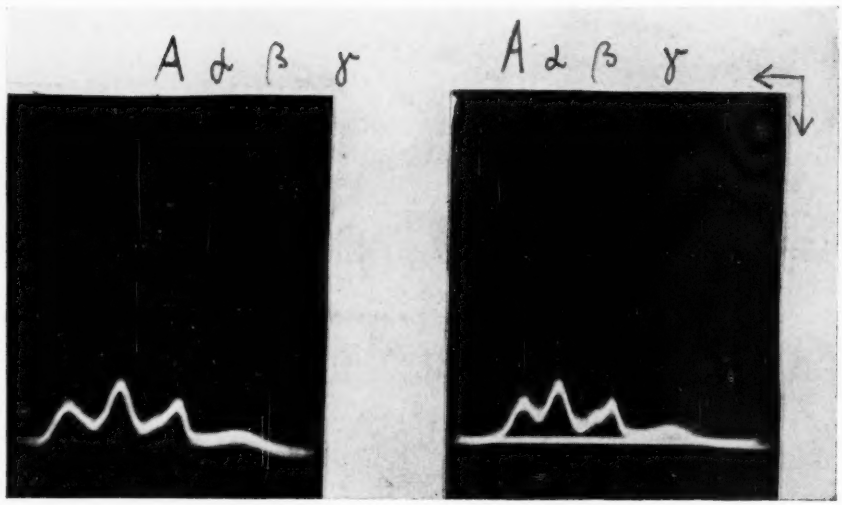
This observation applies similarly to the globulin subfractions and is confirmed by the electrophoretic diagram before and after performance of the cadmium reaction (Fig. 1). The fairly regular diminution of all peaks is easily recognizable.

In the myeloma Serum 3 (Table I) where three-fourths of the serum proteins consist of gamma globulin, the cadmium precipitate has a corresponding imbalance (Fig. 2). Probably this would have been even more pronounced if it had not been necessary for technical reasons to keep the total protein content for the three electrophoreses constant. The relative increase of albumin content is thus also explained; however, calculation shows that the albumin in Serum 3 participated only 6.1 per cent in the diminution. In Sera 2 and 1, where the albumin contents were twice and three times that in Serum 3, the proportional figures of the diminution were 3 and 6.4 times as much albumin. Thus in the case of gamma myeloma (Serum 3) less albumin is precipitated than in the other sera. Moreover, in Serum 2, where the albumin and gamma globulin contents were nearly the same, the decrease in albumin amounted to 18.6 per cent, yet that in gamma globulin was 33.0 per cent. Hence it follows that the cadmium reagent precipitates more gamma globulin than albumin.

Alpha and beta globulin are precipitated to a smaller extent, although further investigations are necessary to decide which of these subfractions is predominantly included.

Summarizing, electrophoretic analysis of three pathologic sera with strongly positive cadmium reaction leads us to conclude that the cadmium reagent precipitates gamma globulin primarily, but that alpha and beta globulin and albumin are also included in the precipitate. In this respect the composition of the initial serum, which is relatively dependent on disease, is of decisive importance. In general, the higher the content of one subfraction as compared with the others, the more strongly is it affected by the reagent. Since it is first of all a question of an anion action (the SO_4 ion having a strongly dehydrating action according to its position in the lyotropic group) all protein fractions are affected. Thus the cadmium reagent differs greatly from the thymol and cephalin-cholesterol reagent.

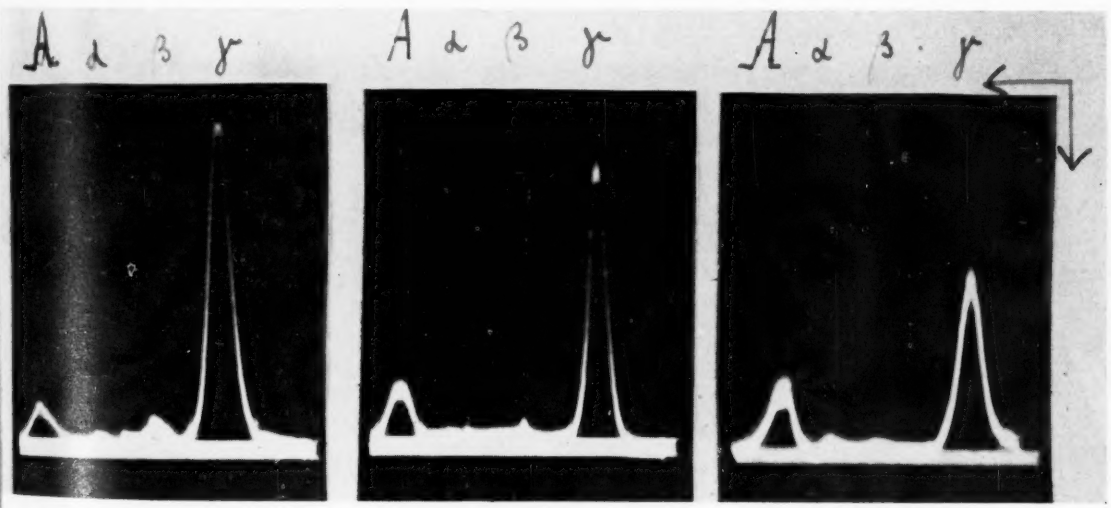
According to Recant, Chargaff, and Hanger[7] (1945), increased lipoproteins lead to a positive thymol test, though they influence the cephalin test but little; they state that the latter reacts considerably to an increase of gamma globulin, whereas thymol shows little or no reaction. Cohen and Thompson[16] (1947) obtained similar results and found a strong thymol turbidity each time the lipoid-carrying beta globulins were markedly increased. This correlation was supported also by electrophoresis after centrifugation of the precipitate, which forms after standing overnight. Since then, Kunkel and Hoagland[8] (1947) have shown by electrophoresis of three thymol-positive sera that this reagent combines chiefly with beta globulin, and to a lesser degree with gamma globulin. In this case also the relative quantity precipitated is in close relation to the protein composition of the initial serum. The complex composition of the precipitate shows that probably our grasp



a Serum: 5.3%

b Serum: 3.8%

Fig. 1.—A 28-year-old woman: pulmonary tuberculosis; amyloidosis of the kidneys.
a, Native serum.
b, Serum after precipitating with $CdSO_4$: equal decrease of every fraction; the total diminution of the serum proteins is 30.2 per cent or 1.52 Gm. per cent (see Table II).
Electrophoresis: descending boundaries
Temp. 3° Cel.; pH = 7.9; μ = 0.1; Buffer: Michaelis barbiturate



	a	*b*	*c*
Total protein content:	13.5%	12.7%	10.7%

Fig. 2.—A 62-year-old woman: multiple myeloma (see Table II).
Effect of the $CdSO_4$ reagent:
a, Native serum.
b, Serum after $CdSO_4$ precipitating: decrease of the γ-globulin fraction.
c, Serum after repeated $CdESO_4$ precipitating: further decrease of the γ-globulin fraction.
Electrophoresis: descending boundaries
pH = 7.9; μ = 0.1; temp. 3° Cel.; Buffer: Michaelis barbiturate

of these reactions between reagent and protein subgroups is not yet complete. Verification of the precipitation optima by Maclagan and Bunn[9] (1947) on electrophoretically-isolated protein fractions suggested, however, a greater susceptibility of the cephalin-cholesterol reagent to gamma globulin. This was found to be increased about ten times with gamma globulin taken from a hepatitis serum. This is at variance with the findings of Kabat, Hanger, Moore, and Landow[10] (1943) who observed a similar precipitation with the cephalin test, and also with the colloidal gold test, even when the gamma globulin had been isolated from different patients. Maclagan[18] found no reaction between isolated alpha and beta globulins and the thymol reagent. He concludes that the latter reacts with gamma globulin. Further investigations are necessary to clear up this question, including the intense yet varying inhibitive action of albumin (Kunkel and Hoagland[8]) and the alterations in precipitative ability of the individual reagents in disease connected with their varying response in the course of the illness. It also has been proved that during convalescence the individual tests remain positive for varying lengths of time. (See Gray and Barron;[11] Martin;[12] Maizels.[13])

For clinical needs simultaneous application of several tests to the same case is to be recommended at present. As we have already shown (Wuhrmann, Wunderly, and Bubb[14]) the average response to the cephalin and thymol reaction agrees to a large extent in extensive series of tests on three different disease groups (hepatic disorders, infectious diseases without participation of the liver, pulmonary tuberculosis). This is true also when the cadmium test is included in such a comparison. (Table II.) On the other hand, parallel tests performed simultaneously often exhibit divergences, sometimes one, sometimes another test appearing more sensitive. These individual deviations are only equalized, as Table II shows, to a very considerable extent, when results are taken as a whole.

TABLE II

	HEPATIC DISORDERS	INFECTIOUS DISEASES (WITHOUT PARTICIPATION OF THE LIVER)	PULMONARY TUBERCULOSIS
	NUMBER OF CASES EXAMINED		
	180	135	106
Positive result:			
Cephalin test	126	84	55
Thymol test	131	82	60
Cadmium test	123	93	68

We have already drawn especial attention to the significant fact that under pathologic conditions a globulin increase (of one or more subfractions) together with a simultaneous albumin decrease is always observed in every case, and that among many thousands of investigations we never observed an albumin increase. The observation of this one-sidedly inverse regulation mechanism is thus of especial importance to the result of serum lability tests, since the positive result of these tests is dependent on an increase of one or

several globulin fractions, the albumin always appearing diminished at the same time. Precipitation of the albumin by the cadmium reagent never occurs in normal plasma under the test conditions described; in the case of disease-occasioned alterations in the serum, where precipitation of the albumin may occur by means of the cadmium reagent, the albumin is always quantitatively diminished. In exactly the same way, the quantitative decrease of the albumin always also appears to be connected with qualitative alterations, as is observed in the increase of the globulin fractions connected therewith. It remains to be seen whether in disease the two fractions of albumin, as they were shown by Luetscher[17] (1939) at pH 4, have different ratios from normal serum; an increase in one of them might easily lead to a different reactivity with the reagents of the lability tests. As a rule albumin does not split up into several underfractions at the pH range in which electrophoresis is usually carried out, and we are, therefore, not well informed on the changes in the albumin fraction. According to our experience also, the lipoid content of the serum (and thus the beta globulin complex) plays a particular part in the thymol turbidity test. (Kunkel and Hoagland,[8] Hanger.[15])

The view that an actual specificity exists in all these tests in respect to hepatic diseases or parenchymatous damage to the liver finally has been abandoned (Wuhrmann, Wunderly, and Bubb[14]). For the cadmium reaction we have emphasized from the beginning that clinical evaluation must make a strict distinction between afebrile disease (positive result especially in *hepatic* and *renal* diseases) and fever states in which the positive result is, generally speaking, an expression of the reaction of the reticuloendothelial system. Table II shows that this observation also applies to the cephalin-cholesterol and thymol tests.

SUMMARY

1. The cadmium reaction, a simple test utilizing a stable reagent and five-minute reading, is described. According to electrophoretic tests it is not dependent on one but on several protein fractions and especially on their relationship with one another. The positive cadmium reaction is due, above all, to an increase of the gamma globulin, but an increased content of the alpha and to a lesser extent of the beta globulins contributes. Precipitation of the albumin is likewise observed in such sera.

2. The greater the increase in content of an individual globulin fraction, the more will be precipitated by the cadmium reagent. The albumin fraction precipitates only if the globulin fractions exhibit morbid displacement.

3. Attention is drawn to the clinical importance of the cadmium reaction and its delimitation in respect to the cephalin-cholesterol and thymol reactions.

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AN IMPROVED DEVICE FOR OBTAINING PLASMA ANAEROBICALLY

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THE method of centrifuging blood in the syringe in which it is drawn in order to obtain plasma anaerobically* has proved useful in research and clinical laboratories. When the syringe is centrifuged the barrel is prevented from being forced over the plunger by means of a metal or plastic spacer held

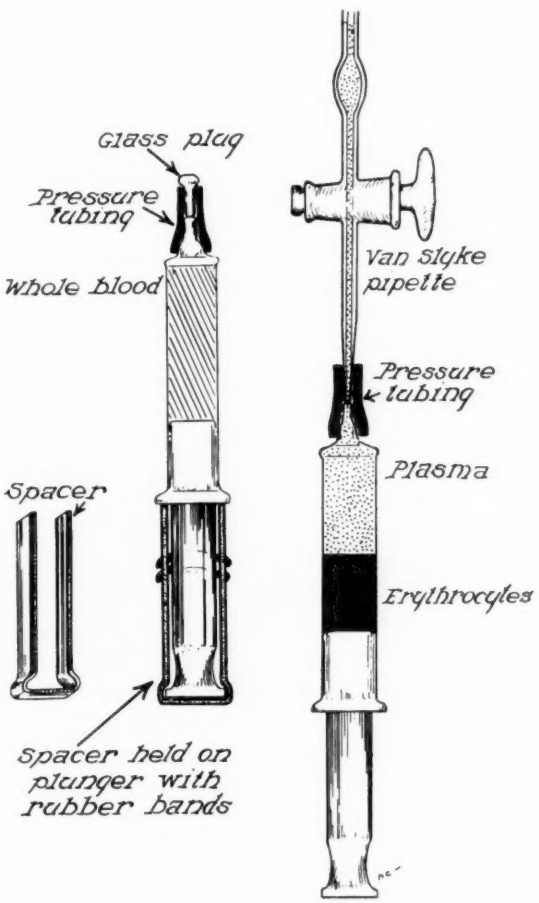

Fig. 1.—The use of the syringe spacer to obtain plasma anaerobically.

on the plunger by rubber bands. However, construction of the spacers as originally described requires the services of a moderately skilled mechanic. The improved spacer described here can be made by anyone.

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*Davenport, H. W.: A Simple Anaerobic Method of Obtaining Plasma, Science **105**: 2717, 1947.

DESCRIPTION

The spacer is made of a 1 cm. wide strip of 20 gauge aluminum or other metal bent into the shape shown in Fig. 1. The strip of metal can be shaped by bending it around two nails driven in a board. It is advisable to make spacers of graded sizes. The most useful sizes are those which when placed over a 5 ml. syringe will permit it to hold 5, 4, or 3 ml. of blood. Spacers for 2 ml. syringes are particularly useful when blood is being drawn from small animals.

The entire plunger of the syringe is greased with a light-bodied stopcock lubricant. The plunger is pushed into the barrel and turned through several revolutions. A needle is placed on the syringe, and a few drops of heparin solution are drawn into it. If more than a small bubble of air remains in the syringe it is expelled. Blood is drawn into the syringe, and some is expelled for measurement of pH. The needle is removed, and a length of pressure tubing about 1.5 cm. long is placed on the tip of the syringe. With the tip held upward the plunger is forced in to fill the lumen of the tube with blood. A glass plug, fire polished on one end and flattened into a knob on the other, is inserted into the lumen of the tubing.

The appropriate spacer is placed around the plunger and tightly wrapped with rubber bands. The plug is removed, and the plunger is pushed in until the spacer comes to rest against the barrel. While the spacer is held in this position the plug is replaced. The assembly is then centrifuged with the plunger down. When centrifugation is finished the syringe is held with tip up and the spacer is removed. The plug is withdrawn and plasma is forced into a blood gas pipette whose tip is inserted into the lumen of the tubing.

ON THE DETERMINATION OF PROTEIN IN SERUM AND IN FRACTIONS OBTAINED FROM SERUM WITH A BIURET REAGENT PREPARED WITH SODIUM HYDROXIDE

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IN 1942 Kingsley proposed the use of a biuret reagent containing 11.7 and 17.3 per cent sodium hydroxide for the determination of total protein and albumin, respectively, in serum.[1] Gornall and co-workers have recently reported that the reagent prepared in this way may lead to serious error and they suggest the use of tartrate as a stabilizing agent.[2] Other workers have applied the biuret reaction in various ways.[3, 4] For the past two years we have employed the reagent of Kingsley with 11.7 per cent sodium hydroxide for the determination of total protein in serum and of the protein remaining in solution after the precipitation of globulin fractions with either sodium sulfate or methyl alcohol.[5, 6] Our experience with this reagent is quite satisfactory and we endeavor to show in this paper that it permits a convenient method for clinical laboratories.

EXPERIMENTAL

The method of Kingsley was modified slightly in order to use the same reagent for serum and for filtrates obtained from serum. The reagent was prepared by adding 1,200 ml. of 1 per cent copper sulfate to 6,000 ml. of a solution containing 968.4 Gm. of sodium hydroxide, using sodium hydroxide pellets of ACS grade from only one source* and copper sulfate, ACS fine crystals,* from only one batch. In contrast to the experience of Gornall and co-workers, we have found the reagent prepared in this way to be stable in a stock bottle for several months and to be uniform on repeated preparation. Standard curves obtained from serial dilutions of serum or filtrates of serum of known protein content, determined by the Kjeldahl method and correction for nonprotein nitrogen, have been identical. Although we have not succeeded in finding a convenient substance which gives the biuret reaction to substitute for serum in the standardization, Hiller, Grief, and Beckman have reported that chromium ammonium sulfate has essentially the same absorption curve as that of the biuret color.[7] Apparently this is not true of the color obtained with the reagent containing 11.7 per cent sodium hydroxide. Fig. 1 compares the curves obtained with an Evelyn colorimeter of chromium ammonium sulfate, cobaltous sulfate, and potassium permanganate. Although neither the chromium complex nor cobalt sulfate is similar to our biuret color, potassium permanganate is quite similar and may be used for standardization. Fig. 2 shows that the color of permanganate obeys Beer's law over a considerable range at 540 millimicrons.

The determination was made as follows. For total protein, 0.15 ml. of serum was added to 10 ml. of the reagent in a colorimeter tube. About 2 ml. of ether were added, and the mixture was thoroughly shaken and allowed to stand at room temperature for fifteen minutes. The color was then measured in an Evelyn colorimeter at 540 mμ, using a blank solution containing 10 ml. of reagent and 0.15 ml. of 0.9 per cent sodium chloride. For albumin in the methyl alcohol filtrates obtained by the method of Pillemer and Hutchinson,[8] 1 ml. of solution was added to 10 ml. of reagent and the color was measured after fifteen minutes, using a blank solution containing all of the reagents. For filtrates

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obtained from serum with various concentrations of sodium sulfate, 3 ml. were added to 10 ml. of the biuret reagent and the color also was measured after fifteen minutes, using a blank solution containing all of the reagents.

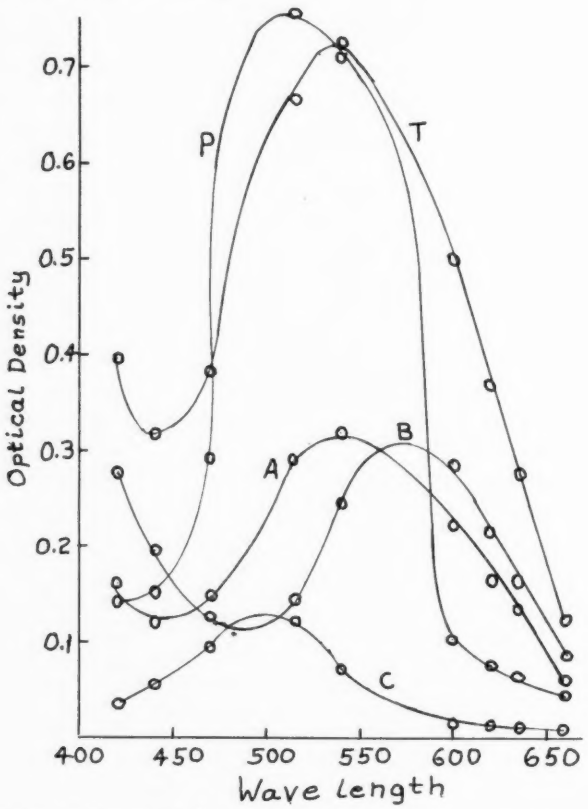

Fig. 1.—Absorption curves.
T, Serum; *A*, filtrate of serum in 21 per cent sodium sulfate; *B*, 0.5 per cent chromic ammonium sulfate; *C*, 0.39 per cent crystalline cobaltous sulfate; *P*, 0.00088N potassium permanganate.

The concentration of protein was calculated from the standard curves which are described by the following equations, in which D is the optical density at 540 millimicrons.

Total protein in serum : $\frac{D}{0.088} \times 100 =$ per cent

Methyl alcohol filtrates : $\frac{D-0.004}{0.1145} \times 100 =$ per cent

Sodium sulfate filtrates

21 per cent* : $\frac{D-0.01}{0.072} \times 100 =$ per cent

26 per cent† : $\frac{D}{0.0694} \times 100 =$ per cent

15, 16, and 19 per cent† : $\frac{D + 0.012}{0.073} \times 100 =$ per cent

*Concentration of the final mixture obtained by adding 0.5 ml. of serum to 9.5 ml. of sodium sulfate reagent.

†Concentration of the final mixture obtained by adding 0.5 ml. of serum to 10 ml. of sodium sulfate reagent.

The filtrate from the method of Pillemer and Hutchinson with methyl alcohol was added at refrigerator temperature, and the standard curve was prepared by also adding samples of filtrate at this temperature. The sodium sulfate filtrates were added at incubator temperature, but the determination in 21 per cent salt

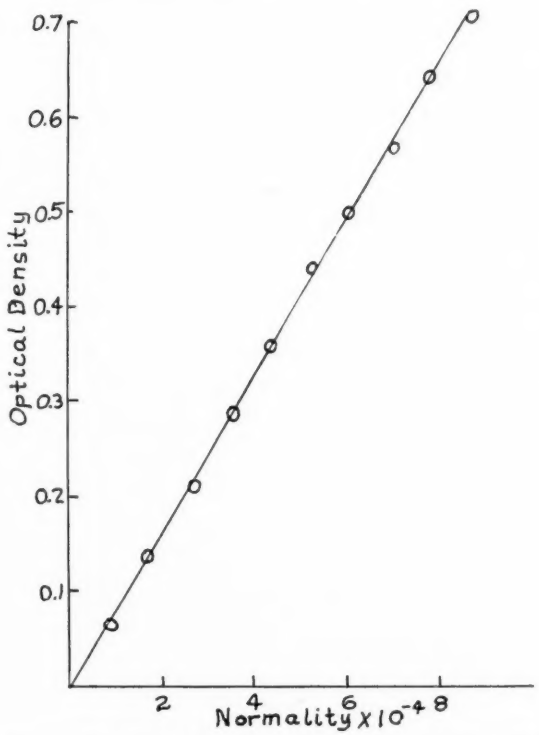

Fig. 2.—Values of optical density for different concentrations of potassium permanganate.

was also made at room temperature. Standard curves were prepared at both temperatures and were found to be essentially the same when the concentration of the reagents of sodium sulfate was adjusted either to room temperature or to 37° C.

Although the temperature of the room is not critical, the results may be affected significantly in warm weather. Table I shows the values of optical density in a series of identical samples which were determined in a water bath at 25° and 30° C. During the summer months it is our practice to keep the tubes in a water bath at 25° until measurement in the colorimeter.

A final remark may be made concerning the determination of protein by the biuret reaction. Our experience confirms that of Wokes and Still that the intensity of the biuret color may be markedly increased in aged samples of serum.[9] We occasionally have found an apparent concentration of as much as 10 and 11 per cent in samples which were stored in the refrigerator for about six weeks. We do not have an explanation for the change which takes place, but it is probably due to bacterial action. There was no increase in the apparent concentration of normal serum that was drawn and stored in the refrigerator for

TABLE I. MEASUREMENT OF THE BIURET COLOR IN SERUM AT 25° AND 30° C.

SAMPLE	OPTICAL DENSITY		APPARENT CONCENTRATION	
	25°	30°	25°	30°
	D		GM. PER 100 ML.	
1	0.653	0.683	7.42	7.76
2	.716	.739	8.14	8.40
3	.520	.530	5.91	6.02
4	.602	.620	6.84	7.05
5	.615	.638	7.00	7.25
6	.629	.663	7.15	7.54
7	.615	.634	7.00	7.21
8	.611	.638	6.95	7.26
9	.602	.620	6.85	7.05
10	.699	.716	7.95	8.14
11	.611	.634	6.95	7.21

six weeks under sterile conditions. However, an apparent change from 7.5 to 8.5 per cent protein took place in the same serum when the flasks were opened occasionally without sterile precautions.

SUMMARY

A biuret reagent prepared according to Kingsley with 11.7 per cent sodium hydroxide is shown to be stable and is recommended for the determination of protein in serum and in both methyl alcohol and sodium sulfate filtrates of serum. Absorption curves are presented for the biuret color obtained with serum and with a sodium sulfate filtrate of serum, and for chromic ammonium sulfate, cobalt sulfate, and potassium permanganate. The curve of the latter most closely resembles that of the biuret color, and this substance is recommended for the standardization of the reagent.

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